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| (54) Title: A FAMILY OF ORGANIC ANION TRANSPORTERS, NUCLEIC ACIDS ENCODING THEM, CELLS COMPRISING THEM AND METHODS FOR USING THEM | | | |
| (57) Abstract The present invention provides a novel family of organic anion transporters of which until now only one member was known. The family includes multispecific organic anion transporters related to the canalicular multispecific organic anion transporter. The cDNA encoding the latter is also provided. The rat and human cDNA are exemplified. Uses of nucleic acids based on this gene family and of cells comprising such nucleic acids as well as vectors comprising sequences thereof are also disclosed especially in the area of gene therapy. | | | |

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Title: A family of organic anion transporters; nucleic acids encoding them, cells comprising them and methods for using them

The present invention lies in the field of molecular biology and genetic engineering. It is particularly concerned with mechanisms of transport for substances across cell membranes. More in particular it is concerned with transport of cytotoxic substances from the inside to the outside of cells.

A group of proteins involved in transport of molecules across membranes is the group of so-called ABC-transporters (ABC: ATP-binding cassette). One member of this group called MRP1 (multidrug resistance-associated protein) has been identified as being involved in transporting organic anions across cell membranes. This protein thus transports different substances than the P-glycoprotein encoded by the MDR-1 gene (MDR: multidrug resistance). MDR-1 is involved in the occurrence of multidrug resistance of for instance tumor cells. Multidrug resistance is one of the major problems in chemotherapy of cancer. On the other hand providing cells with multidrug resistance may be very useful in rescuing for instance bone marrow when chemotherapy is applied. Thus on the one hand there is a need for being able to prevent transport of cytotoxic substances out of the cell while on the other hand there is a need to be able to enhance transport of cytotoxic substances from cells. The P-glycoprotein encoded by MDR-1 is not capable of transport of all cytotoxic substances; its binding specificity is limited to certain groups of molecules. MRP-1 has a different binding specificity in that it transports anionic organic compounds, possibly complexed or conjugated with other substances. In the liver a protein has been characterized functionally which is an ATP-dependent non-bile salt organic anion transporter called canalicular Multispecific Organic Anion Transporter. This protein has been thought to be identical to MRP-1.

The liver plays a major role in the detoxification of many endogenous and xenobiotic, lipophilic compounds. Detoxification is accomplished by transferase-mediated conjugation with glutathione-, glucuronide-, or sulphate-moieties, resulting in negatively charged, amphiphilic compounds which are efficiently secreted into bile or urine. Hepatobiliary excretion of these conjugates is mediated by an ATP-dependent transport system, the canalicular Multispecific Organic Anion Transporter (cMOAT), located in the apical (canalicular) membrane of the hepatocyte (1). The identification of a transport-deficient mutant rat strain, the TR⁻ rat (2), has contributed to the functional characterization of cMOAT (1). These rats have an autosomal recessive defect in the hepatobiliary excretion of bilirubin glucuronides (3) and other multivalent organic anions including, glutathione-S-conjugates (e.g. leukotriene C₄), and 3-OH-glucuronidated and -sulphated bile salts (4). Thus far neither a protein nor a complete cDNA encoding cMOAT have been identified. Transport studies in plasma membrane vesicles from cells overexpressing the human Multidrug Resistance-associated Protein 1 (hMRP1) (5), demonstrated a role for hMRP1 in the ATP-dependent transport of the glutathione conjugates LTC₄ and dinitrophenyl glutathione (GS-DNP) (6). Because these substrates are also transported by the putative cMOAT protein, MRP1 as stated before, is a possible candidate gene for cMOAT. A recent study suggested lateral as well as canalicular localization of the rmrp1 gene product in Wistar liver, but only a lateral localization in TR⁻ liver (14) and suggested a role for MRP1 in the (defective) hepatobiliary excretion of organic anions in TR⁻ rats. In our view, however, the extremely low MRP1 expression in liver (7,5) renders it unlikely that this gene product is responsible for biliary organic anion secretion. Furthermore, the transport defect in the TR⁻ rat appears to be specific for liver (9), while MRP1 is expressed in all human tissues (7). We have now found that cMOAT is encoded

by a different gene and thus that a family of organic anion transporters exists.

The present invention now provides a nucleic acid comprising a sequence encoding at least a part of a member of a family of organic anion transporters, said nucleic acid comprising at least a gene family specific fragment of one of the sequences of fig.1a or fig.1b or figs 17, 18 or 19 or the complement thereof, or a sequence having at least 55%, preferably 70%, in particular 90% homology therewith. Of this family so far only one member, mammalian MRP was known.

We hypothesized that cMOAT might be a liver-specific homologue of MRP1. To obtain a rat *mrp1* probe, we applied the polymerase chain reaction (PCR) on rat lung cDNA using nested degenerate oligonucleotide primers which were based on the highly conserved first ATP-binding cassette of the hMRP1 sequence (see experimental part). The 213 base pair product obtained shared 83% amino acid sequence identity with the corresponding region of the hMRP1 sequence. When analyzed on Northern (RNA) blot, this PCR fragment hybridized with a single, 9.5-kb, transcript in all Wistar and TR⁻ rat tissues examined, with high expression in lung and testis, but no detectable expression in liver. Because this expression pattern resembled that of hMRP1 in human tissues (7), we assumed that we had isolated a part of the rat homolog of hMRP1, rat *mrp1* (*rmrp1*). In order to find the putative *cmoat* gene, two rat liver cDNA libraries were screened, using the *rmrp1* fragment obtained as a probe (see the experimental part). This resulted in the isolation of a full length cDNA with a single open reading frame of 1541 amino acids (Fig. 1b). Based on similarity searches (10), the protein was identified as a new member of the ABC-transporter family (11), with modest identity to other members of the family. Highest overall identity was found with hMRP1 (47.6%) (5), yeast Cadmium Factor 1 (41.8%) (12), and the human Cystic Fibrosis Transmembrane Conductance Regulator (30.2%) (13). The amino acid sequence identity with hMRP1 ranged from 38-61% outside the ATP-binding

domains to 67% and 75% in the first and second ATP-binding domain, respectively. Recently, two different partial *rmpl* cDNA sequences were disclosed (14). One of these sequences comprised a 347 nt fragment that closely resembles the rat

5 *cmoat* cDNA sequence found by us, said partial sequence is, however, not identical to *cmoat*. Moreover, no relation has been made between said partial sequence and the putative cMOAT protein. In contrast, it was suggested that a mutant *mrpl* gene is responsible for the cMOAT-deficient phenotype.

10 Northern (RNA) blot analysis of rat tissues with a 1-kb restriction fragment of our isolated cDNA, revealed three different transcripts, ranging from approximately 6.5 to 9.5-kb, with high expression only in liver, and low expression in kidney, duodenum, and ileum (Fig. 2A). These

15 transcripts were strongly decreased (but not absent) in liver (Fig. 2B) and other tissues of the TR⁻ rat, which suggests that these transcripts were related to the defect in the TR⁻ rat. The three transcripts observed were probably derived from a single gene, because the level of all three

20 transcripts was decreased in the TR⁻ rat. The decrease of this transcript in TR⁻ liver suggests that the isolated cDNA encoded *cmoat*.

To examine the expression level and the cellular localization of the cMOAT protein in hepatocytes, we

25 produced a monoclonal antibody (mAb M₂ III-5) to a bacterial fusion protein containing the 202-amino acid carboxyl-terminal end of the sequence (see experimental part). On protein blots this antibody detected a protein of approximately 200-kD in the canalicular, but not the

30 basolateral plasma membrane fraction of the Wistar rat liver (Fig. 3). This molecular weight was very similar to that of hMRP1 and in good agreement with the predicted molecular weight of the cMOAT protein. The 200-kD protein was completely absent from the canalicular membrane fraction of

35 the TR⁻ rat (fig.3), which correlated with the decreased mRNA level in TR⁻ rat liver (Fig. 2B). Again, this finding was in good agreement with the defect observed in TR⁻ rats

which lack a functional transport system for organic anions in the canalicular membrane.

Thus we have isolated the complete cDNA encoding the cMOAT protein, which is deficient in the TR⁻ rat. Since the
5 *cmoat* mRNA was not completely absent in TR⁻ liver it was possible to also amplify the complete TR⁻ *cmoat* cDNA by PCR (see the experimental part) using various specific primer sets. To identify the nature of the genetic defect in TR⁻ rats, we sequenced the obtained cDNA. This revealed a 1-bp
10 deletion at amino acid position 393, which results in a frame-shift and subsequent introduction of a stop-codon at position 401 (Fig. 4). This deletion results in the destruction of a *Nla*III restriction site which provided a means to quickly confirm the mutation in cDNAs from various
15 tissues (see the experimental part). The very low mRNA expression in TR⁻ rats (Fig. 2B) might be due to the fact that the frame shift causes premature termination of translation and subsequent increased degradation of the mRNA.

20 Our results show a correlation between the *cmoat* gene, the absence of the gene product from the canalicular membrane, and the defined congenital transport defect in TR⁻ rats. In addition to the exclusive canalicular localization of cMOAT (Fig. 3), we have found that hMRP1 is routed only
25 to the lateral domain of the plasma membrane of pig kidney epithelial cells (see experimental part). Our findings thus suggest a differential localization of MRP1 (basolateral) and cMOAT (canalicular) and imply that cMOAT and not MRP1 is involved in biliary organic anion transport. This contrasts
30 to suggestions made in the literature (14). It was also suggested (14) that an isoform of MRP1 exists in rat liver which is derived from the same gene by alternative splicing based on the detection of two different sequences for the second ATP-binding domain and only one for the first ATP-
35 binding domain. Our complete cDNA data, however, show that there are also two different sequences for the first ATP-binding domain in *mrp1* and *cmoat*. In fact, the two cDNA

sequences differ considerably throughout the entire molecule, thus indicating that MRP1 and cMOAT are encoded by two different genes.

We conclude that the MRP homolog, identified here,
5 encodes the canalicular Multispecific Organic Anion
Transporter, and that a 1-bp deletion, resulting in a
truncated, non-viable, protein, is responsible for impaired
transport of organic compounds from liver to bile in the TR⁻
rat. TR⁻ rats have the same phenotype as patients with the
10 Dubin-Johnson syndrome, characterized by mild chronic
conjugated hyperbilirubinemia (15). Isolation of the human
homolog of cmoat is required to elucidate the nature of the
defect in humans. Overexpression of hMRP1 confers resistance
of human tumor cells to a number of cytostatic drugs (16,
15 17), and this resistance is dependent on intracellular
glutathione levels (18). Apparently, both MRP1 and cmoat are
involved in the excretion of organic anions from cells.
Thus, overexpression of cMOAT, like that of MRP1, might also
confer resistance to cancer cells against cytostatic drugs
20 or their metabolites.

Using the rat cmoat gene we also found and isolated the
cDNA encoding the human cMOAT protein. Now that it is known
that these two exist other species of this family of
transporters can be found using the present invention.

25 These transport mechanisms occur throughout the living
world, so family members can be found in bacteria, bacilli,
yeasts and fungi, plants, invertebrae, vertebrae, in
particular in mammals.

In addition to MRP1 and cMOAT (MRP2), other MRP
30 homologs encoding GS-X pumps are present in the human
genome, considering that there are at least four MRP
homologs expressed in *Caenorhabditis elegans* (56). We
therefore searched the Expressed Sequence Tag (EST) library
(57) for putative human MRP homologs, and found three more
35 MRP homologs expressed in humans. We call these new MRP
homologs MRP3, MRP4, and MRP5.

To investigate a possible role of MRP homologs in drug resistance, we examined a large set of (multi)drug resistant cell lines for the (over)expression of *CMOAT*, *MRP3*, *MRP4*, and *MRP5*. We find that especially *CMOAT* expression is
5 elevated in several cell lines, selected for cisplatin resistance, and also in some sublines of the human non-small lung cancer cell line SW1573/S1, selected for doxorubicin resistance. The expression level of *CMOAT* correlates with the cisplatin but not the doxorubicin resistance of these
10 cell lines. Although *MRP3* and *MRP5* were overexpressed in some resistant cell lines, no clear correlation between drug resistance and the expression levels of *MRP3*, *MRP4*, and *MRP5* has emerged from these studies as yet.

Preferred for the purposes of this invention are
15 closely related members of the members identified by the sequences of fig.1a and fig.1b, most preferred those members which transport similar or the same compounds when expressed in a cell, or the closely related family members identified herein as MRP 3, 4 and/or 5. Most preferred is the human
20 *cmoat* gene or its human family members and their products for their usefulness in for instance gene therapy and for their use in preparing blocking agents to the transporting product.

Further embodiments include but are not limited to a
25 vector comprising a nucleic acid according to the invention and suitable means for replication, transduction and/or expression of said nucleic acid.

Preferably such a vector further comprises a gene encoding a therapeutically beneficial protein, which may be
30 any protein having a beneficial effect under certain circumstances such as giving glutathion elevating activity, which enhances transport of anionic complexes or conjugates by the invented transporters.

Such vectors include vectors wherein the gene encodes
35 at least a functional part of a gamma glutamyl cysteine synthetase or a UDP-glucuronosyltransferase.

Other vectors according to the invention include vectors wherein the therapeutically beneficial protein is another multidrugresistance related protein such as MDR1.

The invention further provides a cell comprising a
5 nucleic acid or a vector according to the invention. Said cells may be any cells, preferred are bone marrow progenitor cells, in particular hematopoietic stem cells.

If said vector thus not encode additional desired functionalities apart from the cMOAT activity as disclosed
10 above, said activity may be present on a separate vector to be introduced into said cell.

The invention also provides a method for providing cells with Canalicular Multispecific Organic Anion Transport protein activity, comprising transducing said cell with a
15 nucleic acid or a vector according to the invention, as well as a method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to the invention, comprising increasing the intracellular level of glutathion, glucuronide and/or sulphate. This may be done by
20 contacting the cell with for instance glutathion esters, but also by providing additional genetic material as disclosed above. This may be done by cotransducing UDP-glucosedehydrogenase or sulphotransferase or any other means of enhancing such activity.

25 Thus the invention also encompasses methods for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to the invention, comprising enhancing the conjugating capacity and/or the complexing activity of said cell for sulphate, glutathion,
30 glucuronide and the like.

On the other hand the invention provides a method for reducing Canalicular Multispecific Organic Anion Transport protein activity and/or the multidrug resistance of a cell comprising providing said cell with an antisense construct
35 of a nucleic acid or a vector according to the invention, which antisense constructs are thus also part of the present invention. These methods can be used to block or at least

reduce transport of substances by the transporter protein according to the invention thus reducing resistance of for instance tumor cells to certain chemotherapeutic substances. Other ways of blocking the invented transporter are also
5 part of the invention. These include methods of reducing the level of the conjugating or complexing molecules that enhance transport by the invented transporter. Antibodies to (in particular the extracellular domain of) the transporter.

For even further reducing multidrug resistance of for
10 instance tumor cells said cells can be further provided with an antisense construct derived from another multidrug resistance related protein such as MDR1.

Proteins encoded by a nucleic acid according to the invention or obtainable by expression of a vector according
15 to the invention are of course also part of the present invention, in particular proteins having Canalicular Multispecific Organic Anion Transport protein activity or Canalicular Multispecific Organic Anion Transport protein specific antigenicity comprising at least part of the
20 sequence of fig.4 or being encoded by at least a part of the sequences of MRP 2, MRP 3 or MRP 4 (as given in the accompanying figures) or derivatives thereof having the same or similar function.

In the following a number of uses of the molecules,
25 cells and methods of the invention are disclosed.

The invention enables the use of a nucleic acid according to the invention or a protein according to the invention in the diagnosis of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular
30 Multispecific Organic Anion Transport protein, as well as the use of a nucleic acid according to the invention or a protein according to the invention in the treatment of Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport
35 protein.

Furthermore the nucleic acids according to the invention can be used as a selectable marker gene.

The members of the gene family disclosed herein have several useful applications in the context of gene therapy.

The concept of gene therapy has a very broad range of applications with one common denominator and that is the transfer of additional, new or corrected genetic information into cells which have a genetic or acquired defect. Examples of genetic disorders eligible for gene therapy are cystic fibrosis, Duchenne's Muscular Dystrophy, cancer, Gaucher disease, Crigler Najjar and Dubin-Johnson syndrome. Examples of acquired diseases are cancer, viral and parasitic diseases. In addition, gene transfer can augment the efficacy of conventional therapies. Vehicles for the transfer of genes into target cells and tissues include vectors of viral and non-viral origin. Among the viral vectors murine based retroviruses and human based adenoviruses are the preferred embodiments.

Retroviruses are RNA viruses which efficiently integrate their genetic information into the genomic DNA of infected cells via a reverse-transcribed DNA intermediate as a proviral copy. Integration into the host's genome and the fact that parts of their genetic material can be replaced by foreign DNA sequences make retroviruses one of the more lucrative vectors for gene delivery in human gene therapy procedures, most notably for gene therapies which rely on gene transfer into dividing tissues. Recombinant murine retroviruses have been the vectors of choice since the start of gene therapy and several clinical trials using recombinant retroviruses are ongoing. In order to generate a recombinant retrovirus which carries the cDNA sequence of a particular gene one needs to introduce the retroviral construct into an appropriate packaging cell line. The retroviral construct carries the cDNA of interest and the cis acting elements for packaging and transcription of the viral RNA genome. The packaging cell line provides the trans acting factors needed for packaging: the *gag*, *pol* and *env* genes. Expression of the retroviral construct into the

packaging cell line results in the production of recombinant retroviral particles capable of transducing susceptible target cells and transferring a particular therapeutic gene. The recombinant retrovirus is stably integrated into the target cell genome and conferred to its daughter cells upon cell division.

Adenoviruses are non-enveloped DNA viruses. The genome consists of a linear, double stranded DNA molecule of about 36 kb. Recombinant adenovirus vectors have been generated for gene transfer purposes. Recombinant adenoviruses can be generated by co-transfection of two E1-deleted recombinant adenoviral DNA constructs, one of which comprising the sequences of interest, into an E1-expressing cell line. In contrast to retroviruses, adenoviruses do not integrate into the host cell genome, are able to infect non-dividing cells and are able to efficiently transfer recombinant genes in vivo. These features make adenoviruses attractive candidates for in vivo gene transfer into target cells which are difficult or impossible to treat ex vivo, such as cells of lung and liver.

Although the skilled artisan will be able to employ other vector systems than those exemplified here, such as Adeno Associated Virus (AAV), adenoviruses and retroviruses are preferred embodiments, because of the extensive experience with these viruses in gene therapy concepts.

Vectors comprising nucleic acids encoding and expressing functional members of the family of organic anion transporters disclosed in the present invention are of particular importance for the treatment of diseases caused by defects in these transporters. Examples of such diseases include Dubin-Johnson syndrome, Rotor syndrome and other cholestatic disorders. The human Dubin-Johnson syndrome

The earliest evidence for distinct canalicular transport systems for bile acids and non-bile acid organic anions came with the recognition of the human Dubin-Johnson

syndrome; this is a rare congenital chronic conjugated hyperbilirubinemia. The hepatic clearance of bilirubin and other cholephilic organic anions, like BSP and indocyanine green, is impaired in these patients, whereas bile acid
5 clearance is normal. The urinary excretion of coproporphyrins, metabolic by-products of heme synthesis, is normal but the proportion of coproporphyrin isomer I is increased. The liver histology of this syndrome is characterized by lysosomal pigment accumulation.

10 Preferred target tissues for the genetic treatment of these diseases include the liver, gut and kidney.

Retroviral vectors comprising the nucleic acid sequences disclosed in this invention are constructed as exemplified in EP/95.201211.0 incorporated herein by
15 reference. Recombinant retrovirus supernatant stocks are produced by introduction of the retroviral constructs in appropriate retroviral packaging cell lines. Adenoviral vectors comprising the nucleic acid sequences disclosed in this invention are constructed as exemplified in
20 EP/95.202213.5 incorporated herein by reference. Adenovirus stocks are produced by transfecting the adenoviral construct into appropriate E1 complementing cell lines.

Hematopoietic stem cells (HSC) are the source for life-
25 long production of all mature blood cell types. Therefore, genetic correction of HSC is expected to result in permanent mitigation of the clinical manifestation of inherited and acquired hematopoietic diseases. Of particular interest are Gaucher disease, thalassemia, sickle-cell anemia, AIDS, and
30 others exemplified in WO93/07281. This makes HSC attractive targets for gene therapy. Currently, the established procedure for gene transfer into long-term repopulating HSC relies on the use of recombinant retroviral vectors. However, the gene transfer efficiency into human HSC is
35 insufficient for the treatment of most hematopoietic diseases. This forms the bottle-neck for a broader application of bone marrow gene therapy. Therefore, it is

preferred to provide the recombinant retroviral vector with a marker sequence for positive selection of transduced cells. Selection for the presence of this sequence can be performed in vitro by culturing the transduced cells in the presence of a selective drug. Another approach is to select for transduced cells in vivo, following transplantation of transduced HSC. Both approaches can be taken by inclusion in the recombinant retroviral construct genes encoding transporter proteins conferring resistance to cytostatic drugs.

The members of the family of organic anion transporters disclosed in the present invention are important examples of genes that can be used for this purpose.

Another important embodiment of the present invention is the use of the disclosed members of the family of organic anion transporters to provide the hematopoietic system of cancer patients with resistance to chemotherapeutic drugs. This makes increased dose-intensity in the chemotherapeutic treatment of cancer possible. For most anticancer drugs increasing the dose-intensity results in increased response rates and a higher proportion of cures. Dose-intensity is the amount of drug administered per unit time, and can be augmented either by increasing the chemotherapy dose or by reducing intervals between cycles. Dose-intensive chemotherapy can produce complete regressions and improve survival in patients with historically refractory solid tumors and non-Hodgkin's lymphomas. Dose-response relationships have been demonstrated for many anticancer drugs. The major dose-limiting toxicity of many anticancer drugs is myelosuppression, which thus prevents optimum dose-intensity administration. Severe myelosuppression makes the patient particularly prone to opportunistic infections and is a frequent reason for curtailing chemotherapy before an adequate therapeutic response has been obtained. With a few exceptions (e.g., hormones) most anticancer drugs used in the clinic today, cause moderate to severe bone marrow toxicity

(e.g., vinblastine, epipodophyllotoxins, cisplatin, carboplatin, melphalan, methotrexate, alkylating agents, nitrosoureas, anthracyclines and anthraquinones).

Increasing the therapeutic index of myelosuppressive anticancer drugs by retrovirus-mediated transfer of genes encoding proteins conferring drug resistance is an attractive prospect. A recombinant retrovirus encoding a mutated dihydrofolate reductase (DHFR) that is highly resistant to the anticancer drug methotrexate has been constructed. Infection of murine bone marrow cells with this retroviral vector and subsequent reconstitution of lethally irradiated mice conferred protection from methotrexate-induced marrow toxicity. Furthermore, it has been demonstrated that transfer of the *MDR1* cDNA into drug-sensitive cells can introduce drug resistance, in vitro as well as in vivo. Members of the family of drugs extruded from the cell by the *MDR1* drug pump are e.g. anthracyclines, vinca alkaloids, podophyllotoxins, and colchicine. Etoposide, a commonly used podophyllotoxin of which the dose-limiting toxicity is restricted to the hematopoietic system, is also pumped by the *MDR1* encoded drug pump albeit only poorly. The MDR related drugs have in common that they are lipophilic compounds derived from various natural products. In general, MDR cells are not cross-resistant to alkylating agents (e.g., chlorambucil and cyclophosphamide), antimetabolites (e.g., cytarabine, methotrexate, and 5-fluorouracil), cisplatin, carboplatin or melphalan. The members of the family of organic anion transporters disclosed in the present invention efficiently extrude organic anion compounds from the cell, including GS-DNP and chemotherapeutic agents such as the conjugated forms of cisplatin, carboplatin, etoposide, chlorambucil, and melphalan. This is shown in a nonlimiting example for cisplatin below. Therefore, introduction of the disclosed transporters into the hematopoietic system of cancer patients allows dose-intensification of frequently used chemotherapeutic drugs such as etoposide and are different

from those protected against by *MDR1* or mutant DHFR.
Therefore introduction of members of the disclosed invention
into the hematopoietic system could lead to increased
efficacy of cancer treatment.

5

The invention will be described in greater detail in the
following experimental part.

Experimental

Example 1. Identification and isolation of the rat *cmoat* (*mrp2*).

5

A 213-bp PCR product was obtained from rat lung cDNA after first round amplification with degenerate primers corresponding to amino acid residues 678-648 (forward) and 770-776 (reverse), and subsequent second round amplification with nested primers corresponding to amino acid residues 694-700 (forward) and 760-766 (reverse) of the *hMRP1* sequence (5).

Partial cDNA clones were isolated from a rat hepatocyte cDNA library (23) which was screened with the 213-bp probe according to standard procedures (24). From a 4.5-kb positive clone a 5'-located, 0.6-kb *HphI* restriction fragment was used to screen a *gt10* 5'-stretch rat liver cDNA library (Clontech, Palo Alto). A 0.8-kb overlapping clone was obtained from which a 0.6-kb *AvaII* probe was isolated to rescreen the same library, resulting in the isolation of another overlapping clone. The 5' end of the cDNA was obtained using the anchored PCR procedure [M.A. Frohman, M.K. Dush, G.R. Martin, Proc. Natl. Acad. Sci. U.S.A. 85, 8998 (1988)]. cDNA synthesis was carried out with 5 µg of total RNA isolated from Wistar rat liver and random hexamer primers using Superscript Reverse Transcriptase II. After purification the cDNAs were tailed with a synthetic oligonucleotide anchor sequence using a 5'-RACE kit (Life Technologies, Gaithersburg). Two rounds of nested PCR (96°C, 30sec; 60°C, 30sec; 72°C, 45sec) using an anchor specific primer and two *cmoat*-specific primers (5'-
tgtccagtatcttctgtgagcg-3' (first round), 5'-
aacacgacgaacacctgcttggc-3' (nested)) resulted in the isolation of the missing 5'-sequence. Probes were labeled with [α -³²P]dCTP using random primers. Hybridization of the filters was performed at 65°C in 0.5 M NaPO₄ (pH 7.0), 2 mM EDTA, and 7% SDS (hybridization solution), for 20 hours.

Filters were washed four times in 2x SSC, 1% SDS for 30 min at 65°C, and autoradiographed. Nucleotide sequences were determined by the dideoxy-nucleotide chain method [F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74, 463 (1977)]. The *cmoat* sequence is being submitted to the Genbank database and is available under accession number L49379.

A fusion gene, consisting of the gene for the *Escherichia Coli* maltose-binding protein, and the 3' part of the *cmoat* cDNA corresponding to amino acid residues 1340-1541, was constructed in pMal-c [C.V. Maina et al., Gene 74, 365 (1977)]. The fusion protein was produced in *E. coli* strain JM101 and purified by amylose resin affinity chromatography. Mice were injected three times over six weeks with 200 µg of the purified protein. The first injection was in the presence of Freund's complete adjuvant, and the subsequent boosts in Freund's incomplete adjuvant. Two weeks after the final boost with a glutathione-S-transferase-cMOAT fusion protein, splenocytes were isolated and fused with myeloma cells. Hybridomas were screened by ELISA with the glutathione-S-transferase-cMOAT fusion protein and subsequently tested for positivity in Western blots.

cmoat cDNA was amplified from liver, kidney, ileum and duodenum from both Wistar and TR⁻ rats using primers corresponding to amino acid residues 366-375 (forward) and 451-458 (reverse) of the *cmoat* sequence. The obtained PCR product was digested with *Nla*III. In all PCR products from TR⁻ rat digestion produced two bands of 206 and 66 bp whereas in the Wistar three bands of 83, 122 and 67 bp were observed.

Total RNA was extracted using the acid-phenol single step method [P. Chomczynski and N. Sacchi, Anal. Biochem. 8, 148 (1987)]. Poly(A)⁺ RNA was isolated using the polyAtract mRNA system III (Promega, Madison). RNA was fractionated on a 0.8% denaturing agarose gel, transferred to Hybond N⁺ nitrocellulose membrane filters and hybridized with a [a-

³²P]dCTP-labeled 213-bp rat lung *mrpl* probe and a 1-kb *Hind*III/*Ava*II fragment of *cmoat* in hybridization solution (11) for 20 hours at 65°C. Filters were washed 4x30 min in 0.2x SSC/0.1% SDS at 65°C and autoradiographed. A ³²p-

- 5 labeled 1.2-kb *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA [Ph. Forth et al., *Nucleic Acid Res.* 13, 1431 (1985)] was used to estimate variations in RNA loading.

- Canalicular and basolateral membranes were isolated as described by [P.J. Meier, E.S. Sztul, A. Reuben, J.L. Boyer, *J. Cell Biol.*, 98, 991 (1984)]. Membranes, containing 50 µg of protein were fractionated by 7.5% SDS polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose filters, blocked for at least 15 2h in PBS/M/T (phosphate-buffered saline containing 1% BSA and 1% milk powder and 0.05% Tween-20), and incubated with the monoclonal antibody (M₂ III-5 hybridoma culture medium diluted eightfold with PBS/M/T) for 2h. Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse 20 immunoglobulins and subsequent staining with 3,3'-diaminobenzidine and 4-chloro-1-naphthol substrate. P-glycoproteins were detected using the monoclonal antibody C219 and peroxidase-conjugated rabbit anti-mouse IgG. Immune complexes were visualized by enhanced chemiluminescence 25 detection.

Example 2. Isolation and characterization of the human cMOAT

The human homolog of the rat cMOAT cDNA was isolated using a 4 kb fragment of the rat cMOAT cDNA. The fragment
5 was labelled as described for the rat cMOAT cDNA. The labelled probe was then used to screen a human lambda gt11 liver cDNA library. Three clones with inserts hybridizing with the rat cMOAT cDNA sequence were isolated and designated clone 12, 7 and 20. Clone 12 contained an insert
10 of 2716 nucleotides comprising coding sequence 130-2846. Clone 7 contained an insert of 2000 nucleotides comprising coding sequence 2517-3185. Clone 20 contained an insert of 2231 nucleotides comprising the coding sequence 3069-5300. Missing nucleotides 1-130 encompassing the translation
15 initiation site were obtained from the WashU-Merck EST library, clone 1243479. Furthermore noncoding 3' sequences were found to be present in additional EST clones and were used to complete the full coding sequence of the human cMOAT cDNA. Clones 193244 and 199655 were used for this purpose
20 and completed the full length sequence from 5300 to 5582 nucleotides.

Example 3. Transport experiments with rat cMOAT transfectants.

25 The rat cMOAT cDNA was cloned into the mammalian expression vector pSVK3 (Pharmacia). pSVK3-rat-cMOAT and pSVK3 with rat-cMOAT in the reverse orientation (pSVK3-rat-cMOAT/Rev) relative to the promotor were transfected into COS-7 cells
30 grown in 75 cm² tissue culture flasks. Three days after transfection, the cells were used for GS-DNP transport experiments and analyzed for cMOAT protein expression using anti-cMOAT antibodies. For transport measurements the cells were washed with Hanks buffer and loaded with Hanks/¹⁴C-CDNB
35 at 15°C. Samples were taken after various time points. Input CDNB and cell mediated formation and transport of GS-DNP were separated by extraction of the samples with water-

saturated ethylacetate. The water phase which contains the excreted ^{14}C labelled GS-DNP was counted in a scintillation counter. Total protein was determined using the Lowry assay. GS-DNP efflux from the transfected cells was measured in a scintillation counter. Relative to COS-7 cells transfected with pSVK3-rat-cMOAT/Rev, cells transfected with pSVK3-rat-cMOAT excreted two fold more GS-DNP (See figure 5). This suggests that rat cMOAT transfected cells express a functional organic anion transporter protein in line with the expression of a protein reactive with anti-cMOAT antibodies.

COS-7 cells transfected as described above were also used to isolate membrane vesicles and perform transport experiments. For this purpose cell homogenates were prepared from transfected COS-7 cells and were centrifuged over a discontinuous gradient of 19, 38 and 56 % sucrose. The 38-19 % interface was collected and revesiculated and total protein content was determined using the Lowry method. The vesicle suspensions were incubated with ^3H -GS-DNP at 37°C in the presence of an ATP regenerating system. After the indicated time points the vesicle suspensions were filtered. Then the filters were washed with ice-cold stop buffer (250 mM sucrose, 20 mM HEPES/Tris pH = 7.4) and counted in a scintillation counter. In agreement with the cell transport experiments, vesicles isolated from cells expressing rat-cMOAT exhibited GS-DNP transport above the level of transport observed with vesicles isolated from pSVK3-rat-cMOAT/Rev transfected COS-7 cells. This transport was completely dependent on the presence of ATP characteristic for a member of the ABC transporter superfamily (figure 6).

Example 4: Transport experiments with human cMOAT transfectants

35

We have attempted to express the human cMOAT protein in several mammalian cell lines and in the yeast *Saccharomyces*

cerevisiae with various expression constructs carrying the human cMOAT cDNA. From 80 independent human cMOAT transfected and cloned human S1 cells only 2 clones expressed the human cMOAT protein at low levels. These 5 expressing cells were tested for functional cMOAT by plating the cells in varying concentrations of doxorubicin, vincristin and cisplatinum.

The human LLC-PK1 cell line was also transfected with the same human cMOAT DNA construct and 90 clones were screened 10 for expression. None of these clones expressed the human cMOAT protein as detected with antibody M₂III-6. In contrast, expression of human cMOAT in yeast was also studied and was high after the translational core sequences of the human cMOAT cDNA were converted to yeast consensus 15 sequences.

However in both cases no active cMOAT mediated transport of GS-DNP could be observed. To investigate the role of cellular polarity in determining functional expression of the human cMOAT protein we have infected MDCK cells with an 20 amphotropic retrovirus carrying the human cMOAT cDNA. For this purpose a HindIII-NcoI DNA fragment containing the complete predicted human cMOAT open reading frame was cloned into the retroviral vector pCMV-neo (Bender et al., 1987) resulting in a construct designated pCMV-neo-human- 25 cMOAT. The retroviral amphotropic packaging cell line Phenix kindly provided by G.P. Nolan, Stanford University Medical Center, Stanford, USA) was cultured in Iscove's with 10% fetal calf serum. Phenix cells were transfected with pCMV-neo-human-cMOAT DNA using a commercially available calcium phosphate transfection kit (Gibco/BRL). After 16h at 5% CO₂ 30 , 37 °C, medium was changed and growth was continued for 48 h followed by collection of recombinant retrovirus containing medium. Storage of virus supernatants was at -20 °C. The MDCK cell line strain II (MDCKII; Louvard) was used 35 for transduction experiments. For this purpose 2 x 10⁵ MDCKII cells were seeded and incubated with a 5 ml 1/10 diluted virus stock in medium containing 30 mg Transfection

Reagent (DOTAP; Boehringer Mannheim, Germany). After 10 h medium was replaced with fresh medium. Thirty six hours after infection cells were trypsinized and seeded at dilutions varying between 1/12-1/64. Stably infected cells were selected for 2-3 weeks in medium with G418 at 200 mg/ml. Thirty clones were picked and analyzed for the presence of hcMOAT protein. Western blot analysis of crude membrane fractions of these clones revealed that several clones contained a substantial, but between individual clones variable, amount of human cMOAT. Two of these clones are shown in Figure 7. A weak signal was observed in wild-type MDCKII cells with a slightly higher molecular weight than hcMOAT after prolonged exposure. This might either represent canine cMOAT or another protein to which this mAb cross-reacts. The subcellular distribution of human cMOAT protein was determined in MDCKII-217 hcMOAT transfectants, which showed the strongest signal in Western blot analysis (See figure 7). Cells were grown to confluency on microporous polycarbonate membrane filters (3 mm pore size, 24.5 mm diameter, TranswellTM 3414; Costar Corp., Cambridge, MA) at a density of 2×10^6 cells per well as previously described (8). For confocal laser scanning microscopy, cells were washed in PBS and fixed for 10 min in acetone at rt. Filters were incubated with mAb M₂-III-6 (undiluted) for 60 min. Antibody binding was detected with a FITC-labeled sheep anti-mouse IgG (1:50; Boehringer Mannheim, Germany). Filters were mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing propidiumiodide (1 mg/ml) for counterstaining of nucleic acids. Cells were examined with a MRC-600 confocal microscope (Bio Rad, Hertfordshire, UK). Expression of hcMOAT protein was visualized by indirect immunolocalization using confocal laser scanning microscopy (CLSM). Clear staining was observed in approximately 50% of the cells (Figure 8 A), whereas in MDCKII wild-type only a very weak signal was detected (data not shown). Examination of the cells at the plane perpendicular to the membrane filter revealed that almost all human cMOAT immunostaining

was confined to the apical plasma membrane, although intracellular staining was observed in some cells (Figure 8 B).

To investigate whether human cMOAT expression in MDCK cells allows functional transport of GS-DNP the following experiment was performed. Export of [^{14}C]GS-DNP (GSH dinitrophenyl) from cells was determined by incubating cells with [^{14}C]CDNB ([^{14}C]-1-chloro-2,4-dinitrobenzene: 10 mCi/mmol) as described (8). The resulting hydrophilic GS-DNP only leaves the cell by active transport. Transport of GS-DNP across the apical and the basolateral membrane can be distinguished by growing cells as a monolayer on microporous membrane filters. Briefly, cells were grown on polycarbonate filters (see under immunocytochemistry) for 3-4 days. Two ml of medium (at room temperature) containing 2 mM [^{14}C]CDNB was applied to both the apical and basal compartment of the monolayer and 200 ml aliquots were taken at various time points. After extraction with 200 ml of ethylacetate radioactivity in 160 ml of the water phase was determined by liquid scintillation counting. The amount of radioactivity was corrected for the decrease in volume of culture medium. To determine intracellular radioactivity, cells were washed with cold PBS, filters were cut from the plate and counted directly in liquid scintillation fluid. The resulting pattern of GS-DNP export after exposing MDCKII, and the human cMOAT transfected clones is shown in Figure 9. MDCKII wild-type cells showed a substantial endogenous level of GS-DNP transport. Comparable transport to both the apical and basal compartment was measured in parental cells. Remarkably, apical GS-DNP export was substantially higher in both human cMOAT transfected clones, demonstrating that human cMOAT is active as a glutathione conjugate pump in these cells (figure 9). Comparing the Western blot data (Figure 7) with transport data suggests that there is a correlation between the amount of human cMOAT detectable and the level of apical GS-DNP transport. To exclude that differences in transport capacity between individual clones

were due to differences in GST activity, and therefore differences in conjugation capacity, the total amount of GS-DNP retrieved in cells plus medium after 20 min was calculated. These data revealed that all clones had
5 comparable levels of GS-DNP formed (data not shown).

Example 5: A mutation in the human *cMOAT* gene causes the Dubin-Johnson syndrome.

10 The human Dubin-Johnson syndrome is an autosomal recessive liver disorder characterized by chronic conjugated hyperbilirubinemia. Patients have impaired hepatobiliary transport of non-bile salt organic anions a phenotype similar as has been described for the TR⁻ rat, which has a
15 defective *cmoat*. In view of the identical phenotypes of TR⁻ rats and Dubin-Johnson patients, we have tested whether a mutation in the human *cMOAT* gene also underlies the transport defect in Dubin-Johnson syndrome. In this example we demonstrate that the human homologue of rat *cMOAT*, human
20 *cMOAT* also subject of this invention, is deficient in a patient with Dubin-Johnson syndrome. Furthermore we show that we have used the DNA sequence of the human *cMOAT* cDNA to develop two diagnostic assays for Dubin Johnson syndrome.

We have studied a female, caucasian patient (age 54) who
25 was diagnosed for Dubin-Johnson at the age of 20. She frequently complained of pains in the upper abdomen. General liver function was normal except for elevated conjugated (38 to 70 μ M) and unconjugated (12 to 25 μ M) serum bilirubin levels. It was not possible to visualize the gallbladder
30 after administration of oral contrast reagent, a characteristic feature of Dubin-Johnson. The patient demonstrated a delayed plasma clearance of i.v. injected BSP, followed by a secondary rise in plasma BSP levels. At the age of 32 the patient underwent cholecystectomy. A
35 characteristic black liver was observed and microscopic analysis of a liver section revealed mild fibrosis and the pigment accumulation indicative of Dubin-Johnson. Liver from

this patient was obtained by a needle biopsy. Normal control liver was obtained from surgical pathology specimens.

Biopsies were fixed for histology in 4 % formaldehyde and embedded in paraffin. Skin fibroblasts from the patient and
5 a normal control were obtained by skin biopsy and cultered in Ham F-10 (Life Technologies), supplemented with 10 % fetal bovine serum and antibiotics, at 37 °C.

Paraffin-embedded liver sections of Dubin-Johnson and control liver were examined for the presence and
10 localization of the cMOAT protein, using monoclonal antibody M₂III-6. For this purpose formaldehyde-fixed paraffin-embedded liver sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 0.3 % (v/v) H₂O₂ in methanol for 30 min. Before staining,
15 the sections were pretreated with 0.01 M citric acid (pH 6.0) for 3 x 5 min at 100°C. The sections were blocked with normal rabbit serum for 10 min and incubated with monoclonal antibody M₂III-6 for 1 h. Immunoreactivity was visualized with biotinylated rabbit anti-mouse Fab₂ (Dako Copenhagen,
20 Denmark), followed by streptavidin-conjugated horseradish peroxidase (Dako) in PBS/1% BSA, and subsequent staining with 3,3'-diaminobenzidine tetrahydrochloride and 0.02 % (v/v) H₂O₂ in PBS. P-glycoproteins were detected with monoclonal antibody JSB-1. All sections were
25 (counter)stained with hematoxylin and mounted. The M₂III-6 antibody was produced against a bacterial fusion protein containing the 202-amino acid COOH-terminus of rat cMOAT; it cross-reacts with human cMOAT, but not with human MRP1. In human control liver, like in rat control liver, the antibody
30 stained the canalicular membrane of the hepatocyte. In Dubin-Johnson liver, as in TR⁻ rat liver, no canalicular staining was observed, indicating that this patient lacks the cMOAT protein (figure 11). A positive canalicular staining was observed in both Dubin Johnson syndrome and
35 control liver with JSB-1, an antibody against P-glycoprotein, used as a positive control. To investigate the nature of the genetic defect, total RNA was isolated from

cultured fibroblasts obtained from a skin biopsy of both the patient and a normal control according to the acid-phenol single step method. cDNA synthesis was carried out with 6 µg of total RNA and random hexamer primers with Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV-RT, Life Technologies, Gaithersburg, MD), at 37°C for 1 h, followed by 10 min 65°C to inactivate the M-MLV-RT. The complete *cMOAT* cDNA was amplified by the "touch down" PCR protocol from both patient and control fibroblast cDNA using five sets of *cMOAT*-specific primers:

5'-TAGAAGAGTCTTCGTTCCAGACGCAG-3' (forward I) and
5'-GCAATTTTCAGCAGCTGAGGACTCAC-3' (reverse I),
5'-AAATCCTGGTTGATGAAGGCTCTG-3' (forward II) and
5'-TCCAGGTTACATCTCGGACTCTGGC-3' (reverse II),
5'-ACATCTGCCATTCGAGATGACTGC-3' (forward III) and
5'-CAACTCTCATGTCCCTCTGAGATGC-3' (reverse III),
5'-TGAAGTTCTCCATCTACCTGGAGTACC-3' (forward IV) and
5'-GATGATGGTCAGCTTCTCTCGGAGG-3' (reverse IV), and
5'-GTCATCCCTCACAACTGCCTCTTCAGAATCTTAG-3' (forward V) and
5'-CTGCTAGAATTTTGTGCTGTTACATTC-3' (reverse V). PCR reactions were carried out in a Perkin Elmer GeneAmp PCR system 2400, in 1x *Taq* polymerase buffer (Life Technologies), 1.5 mM of MgCl₂, 0.5 mM of dNTPs, 400 nM of each primer, and 0.5 units of *Taq* polymerase. The PCR products were obtained after application of the "touch down" PCR protocol; the reactions were denatured at 96°C for 5 min, followed by five times 2 cycles with annealing temperatures of 72, 70, 68, 66, and 65°C respectively, and subsequent 30 cycles with an annealing temperature of 64°C. Each cycle started with 20 s at 94°C, 30s at the indicated annealing temperature, and 90s at 72°C. The PCR reaction was terminated after an extension step at 72°C for 10 min. PCR fragments obtained from fibroblasts were excised from agarose gel, purified, ligated into the TA-cloning plasmid pCRTMII (Invitrogen, Leek, The Netherlands), and transformed into INVaF' competent cells (Invitrogen). White colonies were picked, grown overnight, and plasmid DNA was isolated using the alkaline lysis

method. Nucleotide sequences of 5-8 pooled clones were determined by the dideoxynucleotide chain method. Sequence analysis of multiple independent clones revealed a mutation in the patient at codon 1066 (CGA to TGA; arginine to stop-codon) (figure 12), which leads to premature termination of *cMOAT* protein synthesis, the normal protein being 1545 amino acids long (see also figure 10). The mutation results in the loss of a *TaqI* restriction site, and we have confirmed the absence of this site in the patient by *TaqI* digestion of her *cMOAT* cDNA (figure 13). From this observation we conclude that this patient is either homozygous for the mutation in codon 1066, or that the second allele does not give rise to a mRNA.

In conclusion, the identification of a mutation in human *cMOAT* in a patient with the DJS confirms the hypothesis that the TR⁻ rat is an animal model for Dubin Johnson syndrome and provides additional evidence that the *cMOAT* gene encodes the major transporter for organic anions in the liver canalicular membrane. Our demonstration of a low, but detectable expression of the *cMOAT* gene in fibroblasts in addition to a nucleic acid based diagnostic assay for Dubin-Johnson syndrome, allows a simple identification of this inherited disorder, without the need for liver biopsy.

Example 6: Identification and isolation of other members of the family of anorganic anion transporters.

6.1 Materials and Methods

6.1.1 Cell lines

All cell lines used in this study have been described in the literature before: the drug-sensitive and doxorubicin-selected MDR sublines of the non-small-cell lung cancer cell lines SW1573/S1 and COR-L23 (58-61); the small cell lung cancer cell line GLC₄ (62); the lung adeno carcinoma cell line MOR/P (61); and the leukemia cancer cell line HL60

(63); the T24 bladder carcinoma cell line and three CDDP-resistant sublines (45); the 2008 ovarium carcinoma cell line, two CDDP-resistant sublines and a Cd^{2+} -resistant subline (40, 41); the A2780 ovarium carcinoma and the HCT8 colon carcinoma cell lines and CDDP-resistant sublines of both (39, 47); the PXN94 ovarium carcinoma and the tetraplatin-resistant subline PXN94tetR (42); the GCT27 testicular carcinoma cell line and the CDDP-resistant subline GCT27cisR (44); the KB-3-1 epidermoid carcinoma cell line and a CDDP-resistant subline KCP-4 (37, 38). All cells were grown in DMEM or RPMI medium (Gibco, BRL), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units/ml) and streptomycin (50 $\mu\text{g}/\text{ml}$). All cells were free of *Mycoplasma* as tested by the use of the Gene-Probe rapid *Mycoplasma* detection system (Gen-Probe, San Diego, USA).

6.1.2 Clonogenic survival assays

The drug sensitivity of cells was determined in clonogenic survival assays in the continuous presence of drugs. Five hundred cells per well were seeded in 24-well plates and incubated for 24 hrs at 37°C . Drugs, of which concentrations were varied in 2-fold steps, were added and cells were incubated for 5-6 days at 37°C . After this the cells were stained with 0.2% crystal violet in 3.7% glutaraldehyde and colonies containing more than 50 cells were counted. The relative resistance was calculated as the ratio of IC_{50} (Inhibitory Concentration where 50% of the cells survives) of the resistant cell line to the IC_{50} of the parental cell line.

6.1.3 Cloning and sequencing of *MRP3*, *MRP4*, and *MRP5* cDNA

For the isolation of *MRP3*, *MRP4*, and *MRP5* cDNA, human cDNA clones were obtained from the I.M.A.G.E. consortium (64). Additional *MRP3* cDNA clones were isolated by screening a human liver 5' stretch plus cDNA library, oligo(dT) and random primed (Clontech, Palo Alto, USA), using a 1 kb

EcoRI-SacI fragment of a human cDNA clone (no. 84966, Stratagene liver cDNA library #937224) as probe. Several overlapping cDNAs were isolated and sequenced. For *MRP4* the insert of a human cDNA clone (no. 38089, Soares infant brain 1NIB cDNA library) was sequenced, containing the 3'-terminal end of the gene. *MRP5* cDNA clones were isolated by screening a fetal brain cDNA library (Clontech, Palo Alto, USA), using the insert of human cDNA clone (no. 50857, Soares infant brain 1NIB cDNA library) as probe (J. Wijnholds, C. Mol, and P. B., unpublished results). Several overlapping cDNAs were isolated and sequenced. For sequencing the ABI 377 Automatic Sequencer was used. Sequence analysis was done using the GCG package of the Wisconsin University (20). All the sequences have been deposited with GenBank (*MRP3* accession number U83659; *MRP4* accession number U83660; *MRP5* accession number U83661).

6.1.4 RNA

Cytoplasmic RNA from cell lines was isolated by a Nonidet P-40 lysis procedure (24). Total cellular RNA from tissue samples obtained during surgery or at autopsy was isolated by acid guanidium isothiocyanate-phenol-chloroform extraction (65).

25 6.1.5 RNase protections

By PCR amplification of human *cMOAT* cDNA a 241 bp fragment corresponding to nucleotides (nts) 4136-4376 (49; GenBank accession number U49248) was generated. The primers used for amplification were 5'-CTGCCTCTTCAGAATCTTAG-3' (forward primer) and 5'-CCCAAGTTGCAGGCTGGCC-3' (reverse primer). For *MRP3*, *MRP4*, and *MRP5* RNA detection the following fragments were generated by PCR amplification: (i) for *MRP3* a 262 bp fragment was generated using the primers 5'-GATACGCTCGCCACAGTCC-3' (forward primer) and 5'-CAGTTGGCCGTGATGTGGCTG-3' (reverse primer); (ii) for *MRP4* a 239 bp fragment was generated using the primers 5'-CCATTGAAGATCTTCCTGG-3' (forward primer) and 5'-

GGTGTTC AATCTGTGTGC-3' (reverse primer); (iii) for *MRP5* a 381 bp fragment was generated using the primers 5'-GCATAACTTCTCAGTGGG-3' (forward primer) and 5'-GGAATGGCAATGCTCTAAAG-3' (reverse primer). All the fragments were cloned into pGEM-T (Promega, Madison, USA), resulting in the plasmids hcMOAT-241, *MRP3*-262, *MRP4*-239, and *MRP5*-381, and the sequences were confirmed. For RNase protections, a-³²P-labeled RNA transcripts were transcribed from NotI-linearized DNA of hcMOAT-241 and *MRP3*-262, using T7 RNA polymerase, or from NcoI-linearized DNA from *MRP4*-239 and *MRP5*-381, using Sp6 RNA polymerase. For *MDR1* RNA detection, a 301 bp *MDR1* cDNA fragment was used (nt positions 3500-3801 (66)), and for *MRP1* RNA detection a 244 bp *MRP1* cDNA fragment was used (nt positions 239-483 (7)). RNase protections were carried out according to Zinn et al. (67), modified by Baas et al. (58). Protected probes were visualized by electrophoresis through a denaturing 6% acrylamide gel, followed by autoradiography. In all experiments a probe for actin (68) was included as control for RNA input. The amount of *MDR1*, *MRP1*, *CMOAT*, *MRP3*, *MRP4*, or *MRP5* RNA relative to the amount of actin was calculated using a phosphorimager (Fuji BAS 2000, TINA 2.08b).

6.1.6 Protein analysis

Total cell lysates were made by lysing harvested cells in 10 mM KCl/1.5 mM MgCl₂/10mM Tris-HCl, pH 7.4/0.5% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 µg/ml), pepstatin (1 µg/ml), and aprotinin (2 µg/ml). DNA was sheared by sonication and samples containing 40 µg of protein were fractionated by SDS/7.5% PAGE and then transferred onto a nitrocellulose filter by electroblotting. After blotting the filters were blocked for at least 2 hours in Blotto (Phosphate-buffered saline containing 1% bovine serum albumin, 1% milk powder, and 0.05% Tween-20), followed by incubation for 2 hours with the primary antibody in Blotto. cMOAT protein was detected with mouse monoclonal antibodies M₂III-5 or M₂III-6, generated against a bacterial

fusion protein containing the 202 amino acid COOH-terminus of rat cmoat (48). Immunoreactivity was visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark) followed by enhanced chemiluminescence
5 detection (Amersham, U.K.).

6.1.7 Fusion proteins of cMOAT, MRP3, and MRP5.

To test the cross-reactivity of the cmoat monoclonal antibodies with human cMOAT and other MRP homologs, fusion
10 proteins were made of the *Escherichia coli* maltose-binding protein with COOH-terminal ends of human cMOAT, MRP3, and MRP5, respectively, using the plasmid vector pMal-c (69). The expression plasmids encoded, respectively, for cMOAT the 202-amino acid COOH-terminal end, for MRP3 the 190-amino
15 acid COOH-terminal end, and for MRP5 the 169-amino acid COOH-terminal end. The fusion proteins were produced in *E. coli* DH5a and purified by amylose resin affinity chromatography (69).

20 6.1.8 Glutathione assay

Cells ($1-2 \times 10^6$ per well) were plated in triplicate in 6 wells plates in medium with or without drugs. 48 hrs after plating the cells were washed with phosphate-buffered saline and scraped in 10% perchloric acid. Precipitated protein was
25 removed by centrifugation and the supernatant was neutralized by adding 0.5 M MOPS/5 M KOH. The concentration of total glutathione (GSH and glutathione disulfide (GSSG)) was determined according to the recycling method of Tietze (70).

30

6.1.9 Chromosome localizations

For the chromosome localization of MRP3, MRP4, and MRP5, radiation hybrid mapping was performed with MRP3, MRP4, and MRP5 specific primers and two different cell panels,
35 Stanford G3 (StG3; 71) and Genebridge 4RH (Gb4RH; 72). The primers used for amplification were: (i) for MRP3 5'-CTCAATGTGGCAGACATCGG-3' and 5'-GGGAGCTCACAAACGTGTGC-3';

(ii) for *MRP4* 5'-CCATTGAAGATCTTCCTGG-3' and 5'-GGTGTTCAATCTGTGTGC-3'; (iii) for *MRP5* 5'-CCTGTTTGGGAAGGAATATGA-3' and 5'-GGGTCGTCCAGGATGTAGAT-3'. For the PCR reactions 25 ng DNA, 2 ng/ μ l of each specific primer, 0.8 units Goldstar polymerase (Eurogentec, Seraing, Belgium) (*MRP3* and *MRP4*) or 1.5 units Amplitaq Gold polymerase (*MRP5*) were used in a total volume of 25 μ l with 1.5 mM $MgCl_2$ and 100 μ M of each dNTP at final concentrations. The PCR conditions were: initial denaturation 5 min 94°C (*MRP3* and *MRP4*) or 12 min 95°C (*MRP5*), followed by 42 cycles of 15 sec 94°C, 30 sec 58°C, and 45 sec 72°C. Final extension was for 10 min at 72°C. PCR products were resolved by 1 percent agarose gel electrophoresis and the cell line scored positive, negative or ambiguous for presence of the gene. Datafiles were submitted to the Stanford Human Genome Center or Whitehead Institute radiation hybrid mapping databases for placing of the *MRP* genes in context of the respective radiation hybrid map framework markers.

20

6.1.10 Microsatellite repeat analysis

To confirm identity of cell lines and subclones 9 highly polymorphic microsatellite markers were used (D1S1649, D2S434, D2S1384, D3S2427, D9S301, D9S934, D12S2070, D14S611, and D17S969). PCR conditions were as described in the Genome Database (GDB). One primer of each set was labelled with a fluorescent dye and PCR products were visualized by electrophoresis on a ABI 377 automatic sequencer. Data were analyzed with Genetyper software version 1.1.1 (Perkin Elmer, Foster City). Allele sizes were within expected range.

30

6.1.11 deposited clones

The sequences for human *MRP-3*, *MRP-4*, *MRP-5* and for human and rat *cMOAT* are deposited at ECACC (European Collection of Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK) under provisional numbers.

35

96010801-hu-MRP₃ #96A
96010802-hu-MRP₅ #97
96010803-hu-MRP₄ #38089
96010804-hu-cMOAT #33A
5 96010805-hu-MRP₃ #20.11
96010806-hu-MRP₅ #101
96010807-hu-MRP₅ #104
96010808-hu-MRP₅ #105
96010809-hu-MRP₃ #20.1
10 96010810-rat-cMOAT
96010811-hu-MRP₃ #97F

6.2 Results

15 6.2.1 Database search for MRP homologs

We searched human EST databases (dbEST, TIGR) for *MRP* homologs other than *MRP1* and *cMOAT*. Alignment and comparison of EST sequences with homology specific to the 3'-terminal ends of *MRP1* and *cMOAT*, including the coding sequence for
20 the second ATP-binding domain, revealed that there are at least 4 more *MRP* homologs expressed in humans. One of these homologs is the human sulfonylurea receptor (*SUR*) gene (73). The other three *MRP* homologs had not been identified before, and were designated *MRP3*, *MRP4*, and *MRP5*.

25

6.2.2 Cloning and sequencing of *MRP3*, *MRP4*, and *MRP5* cDNA

Additional cDNA clones for *MRP3* and *MRP5* were isolated from a human liver and a fetal brain cDNA library, respectively.
30 *MRP3* and *MRP5* cDNA clones were sequenced as well as the *MRP4* cDNA clone obtained from the I.M.A.G.E. consortium. Both *MRP3* and *MRP5* encode four domain proteins, i.e. proteins with two ATP-binding domains and two domains with transmembrane regions (M.K. and J. Wijnholds, unpublished
35 results). More sequence data will determine whether this is also the case for *MRP4*. Figure 14 shows the protein alignment for the COOH-terminal ends of the various members

of the human MRP family and human SUR. The alignment includes the Walker A and B motifs and the signature sequence of the second ATP-binding domain. The percentages of homology for the COOH-terminal 124 amino acids are shown in table 1. The highest homology is found between MRP1 and MRP3 (86% similarity) and the lowest between SUR and any of the MRPs ($\leq 69\%$ similarity).

6.2.3 Chromosome localization of MRP3, MRP4, and MRP5

The *MRP1* gene has been mapped to chromosome 16 at band p13.13-13.12 (5) and recently the *cMOAT* gene to chromosome 10, band q24 (52, 74). We mapped the other *MRP* homologs on the Gb4RH and StG3 radiation hybrid mapping panels, using *MRP3*, *MRP4*, or *MRP5* specific primers. *MRP3*, *MRP4*, and *MRP5* are located on chromosomes 17, 13, and 3, respectively. The most closely linked markers were D17S797 (Gb4RH) and D17S1989 (StG3) for *MRP3*, WI-9265 (Gb4RH) and D13S281 (StG3) for *MRP4*, and WI-6365 (Gb4RH) and D3S4205 (StG3) for *MRP5*. These results are consistent between the radiation hybrid mapping panels and demonstrate that the new *MRP* homologs are indeed new genes, and not splice variants of *MRP1* or *cMOAT*.

6.2.4 Human tissue distribution of *cMOAT*, *MRP3*, *MRP4*, and *MRP5* RNA

RNAse protections were performed to determine the tissues that express *cMOAT* and *MRP3*, *MRP4*, and *MRP5*. The results are summarized in table 2. Both *cMOAT* and *MRP3* are highly expressed in liver, and to a lower extent also in duodenum. Low expression of *cMOAT* was found in kidney and peripheral nerve. For *MRP3*, substantial expression, similar to expression in duodenum, was also detected in colon and adrenal gland. *MRP4* is expressed at a low level in only a few tissues tested. *MRP5* RNA was detected in substantial amounts in every tissue tested, with relatively high expression in skeletal muscle and brain.

6.2.5 Expression of *MRP* homologs in resistant cell lines

In view of their homology with *MRP1*, *CMOAT* and the three new *MRP* homologs are believed to encode transporter proteins involved in drug resistance. We therefore screened a large set of human cell lines derived from various tissues and their resistant sublines selected with either doxorubicin, cisplatin, tetraplatin, or CdCl_2 . Only resistant lines showing decreased cellular accumulation of drugs were analyzed. All cell lines were analyzed by RNase protection for levels of *MDR1*, *MRP1*, *CMOAT*, *MRP3*, *MRP4*, *MRP5*, and actin RNA. The results are summarized in tables 3 and 4, and an example of each probe is shown in Fig. 15.

High *MDR1* overexpression was detected only in two sublines of the human non-small-cell lung cancer cell line SW1573/S1, both selected for high level doxorubicin resistance (2R160 and 1R500). The low level of *MDR1* RNA in the other cell lines is not remarkable as most of the cell lines selected for our panel were known to have a non-Pgp MDR phenotype. Low *MDR1* overexpression was found in the 2R120, a subline of the SW1573/S1, and in three cisplatin selected sublines of the bladder carcinoma cell line T24. Interestingly, a decrease rather than an increase in *MDR1* RNA was seen in two cisplatin selected sublines of the ovarium carcinoma cell line 2008 (table 4). This phenomenon has been reported earlier in the SW1573/S1 sublines 1R50b, 2R50, and 3R80, selected for low level doxorubicin resistance (58, 59; Table 3).

MRP1 RNA is highly overexpressed in the four non-Pgp MDR cell lines GLC₄/ADR, MOR/R, COR-L23/R, and HL60/ADR, all selected for high level doxorubicin resistance (7, 75, 76). The doxorubicin selected cell lines, derived from the SW1573/S1 cell line, showed no or only a minor increase in *MRP1* RNA, as reported before (7, 77). In the cell lines, selected for cisplatin resistance, we detected no major changes in *MRP1* RNA. Only in two sublines of the T24 cell line, T24/DDP7 and T24/DDP10, and in HCT8/DDP, a subline of

the colon carcinoma HCT8 cell line, a slight (less than 2 fold) increase in *MRP1* RNA was found.

Expression of *cMOAT* varied greatly between the cell lines. Most parental cell lines did not express *cMOAT* or at very low levels. Only the MOR/P and the KB-3-1 parental cell lines showed substantial *cMOAT* RNA levels. Overexpression of *cMOAT* was found in several doxorubicin-resistant sublines of SW1573/S1 (30.3M, 1R50b, 2R120, 2R160, and 1R500), and some cisplatin selected cell lines (2008/C13*5.25, 2008/A, A2780/DDP, and HCT8/DDP).

Similar to *cMOAT*, most parental cell lines either did not express *MRP3* or only at very low levels. The only two parental cell lines, which show high expression of *MRP3*, the MOR/P and the KB-3-1, also show high expression of *cMOAT*. Overexpression of *MRP3* in resistant lines was only found in several doxorubicin-resistant sublines of the SW1573/S1 cell line and the cisplatin resistant HCT8/DDP cell line.

MRP4 is expressed only at low or very low levels in the cell lines we analyzed and no overexpression of *MRP4* was detected in resistant sublines.

MRP5 is expressed in every cell line we analyzed, with the highest levels in MOR/P and 2008, but in none of the resistant sublines *MRP5* is highly overexpressed. Only in three cisplatin resistant cell lines, T24/DDP10, HCT8/DDP, and in the KCP-4(-), a minor increase in *MRP5* RNA was detected.

6.2.6 *cMOAT* protein in resistant cell lines

To investigate whether the increased *cMOAT* RNA levels in the resistant cell lines were accompanied by increased *cMOAT* protein levels, total cell lysates were tested on Western blot with the monoclonal antibodies M₂III-5 and M₂III-6, generated against amino acids 1340 to 1541 of the rat *cmoat* protein (48). To test the specificity for human proteins of the Mabs generated against rat *cmoat*, fusion proteins containing COOH-terminal ends of human *cMOAT*, *MRP3*, and *MRP5*, were made. Both *cMOAT* Mabs, M₂III-5 and M₂III-6,

recognize human cMOAT. M₂III-5 also reacts with the MRP5 fusion protein, and M₂III-6 also reacts with the MRP3 fusion protein. No cross-reaction was detected for both Mabs with MRP1 (data not shown).

5 Protein analysis of the cell lines with the cMOAT Mabs showed the presence of a 190-200 kDa protein in several lines (Fig. 16). Similar results were obtained with M₂III-5 and with M₂III-6 (not shown), indicating that the protein detected is cMOAT. The level of cMOAT protein in each cell
10 line correlated very well with the level of cMOAT RNA, even for the cell lines with only a marginal increase in cMOAT RNA, such as the 2008/C13*5.25 and the 2008/A. The only exception was the cisplatin resistant subline of KB-3-1. KCP-4(-). The Western blot shows that the cMOAT protein
15 level was about 2-3 fold higher in the KCP-4(-) cell line than in the KB-3-1, whereas the RNA levels were the same in parental and resistant cells. Mab M₂III-5 also reacts with MRP5 and MRP5 RNA is raised in the KCP-4(-) cells, but a similar result was obtained with Mab M₂III-6 which does not
20 cross-react with MRP5.

All cell lines with no or only very low levels of cMOAT RNA also contained no detectable cMOAT protein (Fig. 16). The small amount of cMOAT detected in the parental A2780 cell line migrated faster in the gel than the cMOAT protein
25 present in the cisplatin resistant A2780/DDP cell line, or the protein detected in the HCT8, HCT8/DDP, KB-3-1, and KCP-4(-) cells. The varying mobility of cMOAT in the gel could be caused by different degrees of post-translational modification of cMOAT protein in each cell line, as we have
30 observed for MRP1 (77; M.K. unpublished results), but this needs to be verified.

6.2.7 Glutathione assays

In view of the proposed role of cMOAT as a GS-X pump,
35 intracellular GSH levels were measured for the cell lines in table 4. GSH levels were elevated in all resistant cell

lines (Table 4), and were not detectably different in cells cultured with or without drugs (data not shown).

6.2.8 Drug resistance of the cell lines analyzed

5 To determine whether there is a correlation between the elevation of expression of transporters and resistance pattern, we have extended the existing information on these cell lines with a more complete survey of resistance against either cisplatin or doxorubicin (Table 5). Interestingly,
10 all the doxorubicin selected SW1573 cell lines with overexpression of *cMOAT* are also cross-resistant against cisplatin, and the level of *cMOAT* expression correlates quite well with the level of cisplatin resistance (Tables 3 and 5). Cytotoxicity analysis of the KCP-4(-) cell line
15 showed that the IC_{50} for cisplatin for this cell line was much lower than reported (700 nM, RF 1.8 [table 5] instead of 25.000 nM, RF 62.5 [37]), suggesting that this cell line was a revertant or contaminated with another low-level-cisplatin resistant cell line. When these KCP-4(-) cells
20 were cultured in the presence of 6.7 μ M cisplatin, more than 99% of the cells died. The surviving population, KCP-4(+), was highly cisplatin resistant again (IC_{50} 22.400, RF 59 [table 5]), but did not express *cMOAT* anymore (Fig. 16). Microsatellite repeat analysis showed that both cell lines,
25 KCP-4(-) and KCP-4(+), were derived from the parental KB-3-1, indicating that the KCP-4(-) is most likely a revertant.

All cell lines selected for resistance against cisplatin, tetraplatin or $CdCl_2$ are not cross-resistant against doxorubicin (Table 5), with two exceptions: the
30 KCP-4(-) cell line and the PXN94/tetR cell line. Cross-resistance did not correlate with *cMOAT* expression.

6.3. Discussion.

35 6.3.1. The MRP gene family

Our database search of expressed sequence tags has revealed that at least five homologs of *MRP1* are expressed in man.

cMOAT or *MRP2* encodes the major organic anion transporter in the canalicular membrane of hepatocytes (48-52, 55). The product of another homolog, *SUR*, plays a role in the regulation of insulin secretion (73). The other three
5 homologs, *MRP3-5*, are all more related to *MRP1* than *SUR* (Table 1). Identity is highest between *MRP1* and *MRP3* (75%). Since the region taken for comparison is small and one of the most conserved parts of the protein, the overall identity between the *MRP* homologs will probably be lower
10 than the percentages in Table 1.

The *MRP* homologs *MRP3-5* are all located on other chromosomes than *MRP1* and *cMOAT*. This confirms that *MRP3*, *MRP4*, and *MRP5* are not alternative splice products of *MRP1* or *cMOAT*. Klugbauer and Hofmann (78) recently cloned another
15 ABC transporter (*ABC-C*), located in the same chromosomal band as *MRP1*, but this is not a *MRP* homolog, because the identity between these two proteins is only 18%. After our work was completed Allikmets et al. (79) reported the identification of 21 new ABC genes also based on a search of
20 the human EST database and they mapped the identified partial sequences.

6.3.2 Physiological functions of the MRP family members

The physiological role of these new MRP proteins is probably a role in cellular detoxification processes by exporting GSH S-conjugates or other organic anions. GSH S-conjugate carriers have been described in many mammalian cells, including liver, heart, lung, and mast cells and erythrocytes (1B, 80). Kinetic studies indicate that both liver canaliculi and erythrocytes contain two different ATP-dependent transport activities for organic anions (81-84). cMOAT is localized in the canalicular membranes of hepatocytes and the absence of this protein in the TR⁻ rats as well as in a patient with the Dubin-Johnson syndrome shows a role for the cmoat/cMOAT proteins in the transport of non-bile acidic organic compounds from liver to bile (48, 49, 55). The other ATP-dependent transport activity in liver canaliculi, responsible for transport of bile acids from liver to bile is not attributable to cMOAT, because studies with TR⁻ rats and Dubin-Johnson patients showed that bile acid transport was not affected (1B).

Two other congenital liver diseases characterized by a conjugated hyperbilirubinemia, like the Dubin-Johnson syndrome, are Benign Recurrent Intrahepatic Cholestasis (BRIC) and Progressive Familial Intrahepatic Cholestasis (PFIC or Byler disease) (85, 86). The clinical and biochemical features of BRIC and PFIC are suggestive of a defect in primary bile acid secretion (87, 88). BRIC and PFIC have both been mapped to the same region on chromosome 18, 18q21-q22 (89, 90).

In view of the high expression of *MRP3* in the liver (Table 2), *MRP3* may be the bile salt transporter. Since none of the human ABC transporter genes identified thus far maps to chromosome 18 (79; this study) it is unlikely that BRIC/PFIC is caused by a defect in a readily recognizable ABC transporter gene.

GS-X activity has also been found in erythrocytes. Several studies have shown that human and rat erythrocytes

contain a low- and a high-affinity S-(2,4-dinitrophenyl)-glutathione (DNP-SG) transporter (84, 91, 92). The high-affinity DNP-SG transporter is most likely MRP1, since the presence of this protein and its binding to LTC₄ have been shown for erythrocytes (93, 94). The other transporter with low affinity for DNP-SG but high affinity for glucuronides and mercapturates (84) is not cMOAT or the bile salt transporter, because (i) no major alterations in DNP-SG transport in erythrocytes from TR⁻ rats and Dubin-Johnson patients were detected (18), and (ii) erythrocytes transport DNP-SG and GSSG but no bile salts (83). This second transporter may be encoded by one of the other MRP homologs.

6.3.3 Expression of MRP homologs in resistant cell lines

We screened a large set of cell lines and their resistant sublines to see whether MRP1, cMOAT or one of the other MRP homologs is overexpressed. MRP4 was not overexpressed in any of the lines. MRP3 RNA was only found to be elevated in the cisplatin resistant HCT8/DDP cell line and several SW1573/S1 sublines selected for doxorubicin resistance. However, overexpression did not correlate with the level of doxorubicin resistance. For MRP5 low overexpression was found in three cell lines selected for cisplatin resistance (T24/DDP10, HCT8/DDP, and KCP-4(-); Table 4), but many other cisplatin selected cell lines showed no overexpression.

Table 3 shows that the classical non-Pgp cell lines selected for high doxorubicin resistance and known to highly overexpress the MRP1 gene, do not significantly overexpress other members of the MRP family. This is compatible with the interpretation that MRP1 is the transporter responsible for MDR in these cell lines. In the non-Pgp derivatives of the SW1573/S1 cell line presented in Table 3 a more complex situation is found and the contribution of MRP1, cMOAT, MRP3, and the major vault protein, also present at increased levels in some of these cell lines (95), remains to be sorted out.

6.3.4 The involvement of organic anion transporters in cisplatin resistance

Whereas P-glycoproteins do not transport small or highly charged molecules, organic anion transporters, such as MRP1 and cMOAT have been speculatively linked to resistance to oxyanions (arsenite, antimonite) and cisplatin. These compounds can form complexes with GSH and there is now considerable evidence that these complexes are substrates for organic anion transporters. Resistance caused by increased export of these complexes is bound to be complex, as pointed out by Ishikawa (80) and by us (6b, 19, 95). Increased levels of pump or GSH, increased GSH synthesis, or a combination may be required depending on the rate limiting step in drug export.

In the protozoal parasite *Leishmania*, resistance to arsenite and antimonite can be associated with both a 40-fold increase in the *Leishmania* GSH homolog trypanothione (97) and an increase in the MRP-related ABC-transporter PgpA (98). Cancer cells selected for high levels of cisplatin may sometimes also contain extremely high concentrations of GSH (99) and the GSH synthesis in these cells is upregulated (99-101). All of the cisplatin resistant cell lines studied by us have elevated GSH levels as well, albeit not as high as the cell lines isolated by Godwin et al. (99). In contrast to published data, we also find raised GSH levels in the T24 sublines, the GCT27cisR, and the PXN94/tetR cell lines (42, 44, 45). We find no clear correlation, however, between the degree of cisplatin resistance and GSH levels, as observed by Godwin et al. (99). Moreover, all the cell lines studied by us show a decreased accumulation of cisplatin and an organic ion pump may therefore be involved in resistance.

Ishikawa et al. (36) showed that MRP1 is overexpressed in the cisplatin resistant human leukemia cell line HL60/R-CP. They concluded that an increased GSH synthesis in combination with raised MRP1 levels can cause cisplatin

resistance. Active cisplatin efflux has been described in three of the cell lines in Table 4: KCP-4, A2780/DDP, and HCT8/DDP (37-39, 102). The ATP-dependent efflux was inhibited by DNP-SG, indicating that it was catalyzed by a GS-X pump. In addition, the membrane vesicles of the KCP-4 cell line were shown to catalyze an increased uptake of LTC₄ (37, 38), known to be the substrate with the highest affinity for MRP1. However, data from these papers and our study show that MRP1 is not overexpressed in these cisplatin resistant cell lines, suggesting that MRP1 is not the major pump responsible for cisplatin resistance. This is supported by transfection studies with MRP1, which showed no cisplatin resistance of the transfected cells (28, 17). Nevertheless, it remains possible that transport of cisplatin conjugates by MRP1 is efficient and that the low levels of MRP1 present in parental cells suffice for resistance, if formation of cisplatin conjugates in resistant cells is increased, e.g. by an increase in GSH synthesis.

An organic anion pump that is important in cisplatin resistance is cMOAT. Especially striking is the correlation between cisplatin resistance and cMOAT expression in the non-Pgp MDR cell lines derived from the SW1573/S1 cell line (Table 5). These lines were selected for doxorubicin resistance and it is therefore unlikely that other mechanisms of cisplatin resistance are activated in these lines. It should be noted, that these non-Pgp MDR lines, selected for low level doxorubicin resistance, contain multiple alterations in the expression of ABC-transporters. Besides upregulation of MRP1, cMOAT, and MRP3 (Table 3), down-regulation of MDR1 has occurred in these lines (59; this study, Table 3).

Some other cisplatin-resistant lines contain increased levels of cMOAT as well, notably 2008/C13*5.25, 2008/A, A2780/DDP, and HCT8/DDP (Fig. 3, Table 4 and 5). The combination of cisplatin with doxorubicin resistance in resistant cell lines has been reported before (100, 103) and is also present in two other platin selected lines,

studied here, PXN94/tetR and KCP-4(-) (Table 5). All other
platin-selected lines in Table 5 are doxorubicin sensitive,
however. The substrate specificity of the organic anion
pumps in the liver canalicular membrane (cMOAT) and in
5 erythrocytes (presumably mainly if not exclusively MRP1) is
very similar (91). We therefore expect both pumps to confer
similar resistance spectra. We have recently succeeded in
obtaining stably transfected kidney cells in which cMOAT is
properly routed to the plasma membrane (R. Evers, M.K., and
10 P.B.; unpublished). These cells should allow a direct test
of the drug resistance spectrum that can be associated with
cMOAT overexpression.

Overexpression of cMOAT in cisplatin resistant cell
lines was recently also reported by Taniguchi et al. (52).
15 However, in contrast to our results (Table 4) they detected
raised cMOAT RNA levels in the KCP-4 and T24/DDP10 cell
lines. We do not find this. The level of cMOAT RNA was even
decreased in the highly cisplatin resistant KCP-4(+) cells,
and in the T24/DDP10 cell line cMOAT RNA is hardly
20 detectable by RNase protection. We also detect no cMOAT
protein in these cell lines (Fig. 16). Cross-hybridization
of the cMOAT probe used by Taniguchi et al. (52), which
contains the coding sequence of the first ATP-binding
domain, with RNA transcribed from the other MRP homologs
25 might explain the discrepancy. This underlines the
importance of the use of gene specific probes to determine
expression of MRP homologs.

In conclusion, our data and those recently published by
Ishikawa et al. (35, 36), Fujii et al. (37, 38), Goto et al.
30 (39), Chuman et al. (102), and Taniguchi et al. (52) provide
evidence that an organic anion pump, notably cMOAT,
contributes to cisplatin resistance by exporting the
cisplatin-GSH complex. Elevated GSH levels and synthesis may
be required to drive formation of the complex if contact
35 with cisplatin is extended, as is usually the case for cell
lines selected for resistance in vitro.

LEGENDS TO THE FIGURES.

Figure 1a. cDNA sequence of human cMOAT

Figure 1b. cDNA sequence of rat cMOAT

- 5 Figure 2. (A) Northern blot analysis of 2 μ g poly(A)⁺ RNA from Wistar rat tissues hybridized to a 1-kb *Hind*III/*Ava*II cDNA fragment of *cmoat*. RNA was analyzed as described in (the experimental part. Prolonged exposure of the film revealed no detectable expression in other tissues then
- 10 kidney, duodenum, and ileum. (B) Northern blot analysis of 2 μ g of poly(A)⁺ RNA from Wistar and TR⁻ rat liver and hepatocytes hybridized with the same probe as described in (A). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal is shown at the bottom. Molecular size standards are
- 15 indicated at the right.

- Figure 3. Immunoblot analysis of *cmoat* and P-glycoprotein in canalicular and basolateral membrane fractions of Wistar and TR⁻ rat hepatocytes. Lane 1, Wistar basolateral membranes; Lane 2, Wistar canalicular membranes; Lane 3, TR⁻ basolateral membranes; Lane 4, TR⁻ canalicular membranes.
- 20 Upper panel: the blot was incubated with the monoclonal antibody M₂ III-5 directed to *cmoat* (27). This antibody did not crossreact with the hMRP1 protein as tested in total lysates from the MRP-overexpressing cell line GLC4/ADR (20).
- 25 Lower panel: Immunodetection of P-glycoproteins with the Mab C219 in the same membrane preparations. The 150-kD P-glycoproteins are exclusively expressed in canalicular membranes (22). Differential staining of the two fractions demonstrates the separation of the two membrane domains with
- 30 slight contamination of the basolateral fraction by canalicular membranes. Molecular weight markers are indicated.

- Figure 4 Deduced amino acid sequence of the rat cMOAT and alignment with the deduced 70 amino acid sequence of the translated 213-bp putative rat *mrp1* cDNA.
- 35

Figure 5. Transport of GS-DNP in COS-7 cells transiently transfected with rat cMOAT expression constructs. Closed

circles represent cells transfected with pSVK3-rat cMOAT. Open circles represent cells that have been transfected with a pSVK3 construct with the rat cMOAT cDNA in the reverse orientation and serves as a negative control. The results depicted are the mean of three measurements.

Figure 6. Transport of GS-DNP in membrane vesicles prepared from COS-7 cells transiently transfected with rat cMOAT expression constructs in the presence or absence of an ATP regenerating system. Closed squares represent cells transfected with pSVK3-rat cMOAT. Open squares represent cells that have been transfected with a pSVK3 construct with the rat cMOAT cDNA in the reverse orientation and serves as a negative control. The results depicted are the mean of three measurements.

Figure 7. Human cMOAT expression in crude lysates from MDCKII derived transfectants. 2 or 20 mg of total protein was size fractionated in a 7.5% polyacrylamide gel containing 0.1% (wt/vol) SDS. After electroblotting, human cMOAT protein was visualized by staining with mAb M₂-III-6. Protein antibody interaction was detected using the Amersham enhanced chemiluminescence kit (ECL). Lane 1,2 MDCKII cells; lane 3,4 human cMOAT expressing clone MDCKII-216; lane 5,6 human cMOAT expressing clone MDCKII-217. In lanes 1,3 and 5 two micrograms of total protein were loaded and in lanes 2,4 and 6 20 micrograms were loaded.

Figure 8. Detection of human cMOAT in MDCKII monolayers by confocal laser-scanning microscopy. A. Indirect immunofluorescence (FITC) picture with mAb M₂-III-6 on MDCKII-217 cells. Nucleic acids were detected using propidium iodide (red signal). Top view of the cell layer is shown. B. Optical section perpendicular to the plane of the cell layer.

Figure 9. Export of GS-DNP from MDCK-II, MDCKII-216, and MDCKII-217 cells. Cells were incubated with [¹⁴C]CDNB (2 mM) in both the apical and basal compartments. Samples were taken at t = 1, 3, 6, 12, and 20 min from both compartments and extracted with ethylacetate. The amount of [¹⁴C]DNP-GS

excreted (in pmol per 2 ml) was measured and plotted. All experiments were done in duplicate and repeated at least twice. Variation between measurements was below 10%. Dotted line: transport to the basal compartment. Continuous line: transport to the apical compartment.

Figure 10. Deduced amino acid sequence of human cMOAT.

Predicted transmembrane regions are underlined. Walker A, B, and signature sequence are doubly underlined. Predicted N-glycosylation sites conserved in other cMOAT proteins (rat, rabbit) and MRP1 proteins (human, mouse) are indicated with triple asterisks. The triangle indicates the location (amino acid 1066) at which a stop codon is introduced by a C to T transition in DJS cMOAT.

Figure 11. Immunohistochemical detection of the cMOAT

protein in human and rat liver using monoclonal antibody M₂III-6. Sections of a normal human liver (A) and normal rat liver (B), which demonstrate the exclusive canalicular localization of the protein. In liver sections of the DJS patient (C) and the TR⁻ rat (D), no canalicular staining is observed. Magnifications are 20 x (A, C), and 100 x (B, D).

Figure 12. Part of the cMOAT cDNA sequence encompassing the mutation which results in the absence of the functional protein in the patient. The normal sequence is depicted on the right. The arrow indicates the site of the mutation at codon 1066. This codon normally encodes an arginine residue (CGA), but is changed into a stop-codon (TGA) in the patient. The mutation of C to T eliminates the recognition site for the restriction enzyme *TaqI* (5'-TCGA-3').

Figure 13. *TaqI* digest of a part of the cMOAT cDNA that was obtained with primer combination forwardIV/reverseIV. Lane 1 represents healthy control-, lane 2 the patient cDNA digest. Molecular size markers are indicated on the left in kilo base pairs.

Figure 14

Protein alignment of COOH-terminal ends of the five human MRP homologs and human SUR. The alignment was performed with the PILEUP program of GCG (48). The GenBank accession

numbers for the proteins used in this comparison are the following: MRP1 - L05628, cMOAT/MRP2 - U49248, MRP3 - U83659, MRP4 - U83660, MRP5 - U83661, SUR - L78207. The nucleotide binding domain specific signature sequence and the Walker A and B motifs are shown in bold. Asterisks above the alignment indicate identical amino acids in at least four of the five MRP proteins.

Figure 15

RNAse protection assays of RNA transcript levels of *MDR1*, *MRP1*, *cMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5* in the human non-small-cell lung cancer cell line SW1573/S1 and its doxorubicin selected subline 30.3M. 10 µg total cytoplasmic RNA from each cell line was used per probe. The positions of the protected fragments of *MDR1*, *MRP1-5*, and t-actin are indicated.

Figure 16

Immunoblot detection of cMOAT protein in the cell lines analyzed in this paper. Total cell lysates were size fractionated (40 µg per lane) in a 7.5% polyacrylamide gel containing 0.5% SDS. The fractionated proteins were transferred to a nitrocellulose membrane, and cMOAT protein was detected by incubation with monoclonal antibody M₂III-5. The size (kDa) and position of molecular weight markers are indicated.

Figure 17 A/B.

Nucleotide sequence and amino acid sequence of MRP3.

Figure 18.

Partial MRP4 sequence.

Figure 19 A/B

MRP 5 sequences.

Table 1

Homology between the COOH-terminal 124 amino acids of the five human MRP homologs and human SUR. Percentages of identity and similarity were determined using the BESTFIT program of GCG (48).

Table 2

Levels of RNA transcripts of *MRP1*, *CMOAT* (*MRP2*), *MRP3*, *MRP4*, and *MRP5* in human tissues. RNA expression levels were determined by RNase protection assays with 10 µg total RNA from various human tissues per probe. Expression of *t*-actin was taken as control for total RNA input. Data for *MRP1* RNA levels are from Zaman *et al.* (52). The relative expression level is indicated by filled circles, very low or undetectable RNA levels by open circles. nd = not determined.

Table 3

Characteristics of the doxorubicin-selected cell lines analyzed in this paper. Resistant cell lines were selected by chronically exposing them to the concentrations of doxorubicin as shown. RNA levels were determined as in Figure 2. The relative expression level is indicated by filled circles, very low expression by $_$, and undetectable RNA levels by open circles.

Table 4

Characteristics of cell lines selected for resistance to cisplatin, tetraplatin or CdCl_2 . Resistant cell lines were selected by chronically exposing them to the concentrations of drugs as shown. Only A2780/DDP and HCT8/DDP were selected by challenging them 1 h weekly with 50 µM cisplatin. RNA levels were determined as in Figure 2. The relative expression level is indicated by filled circles, very low expression by $_$, and undetectable RNA levels by open circles. Data for total intracellular glutathione concentrations were obtained from three independently isolated cell extracts assayed in three independent experiments using the recycling method of Tietze (56) and presented as the mean GSH \pm SD.

Table 5

IC_{50} values and relative resistance factors (RF) of the cell lines analyzed for cisplatin and doxorubicin. IC_{50} data were obtained from clonogenic survival assays with continuous exposure to drugs. The relative resistance factor was

determined by dividing the IC_{50} of each resistant cell line by the IC_{50} of the corresponding parental cell line. Also shown are the levels of RNA transcripts of *MRP1* and *cMOAT*, taken from Table 3 and 4.

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Table 1

Homology between COOH-terminal 124 amino acids of human MRP homologs and human SUR (% similarity / % identity)

| | MRP1 | cMOAT | MRP3 | MRP4 | MRP5 | HSUR |
|-------|-----------|-----------|-----------|-----------|-----------|-----------|
| MRP1 | 100 / 100 | | | | | |
| cMOAT | 79 / 68 | 100 / 100 | | | | |
| MRP3 | 86 / 75 | 79 / 62 | 100 / 100 | | | |
| MRP4 | 75 / 60 | 75 / 54 | 71 / 53 | 100 / 100 | | |
| MRP5 | 75 / 55 | 75 / 53 | 71 / 51 | 73 / 57 | 100 / 100 | |
| HSUR | 69 / 48 | 68 / 46 | 66 / 46 | 69 / 48 | 67 / 45 | 100 / 100 |

Table 2

Tissue distribution of human *MRP* gene transcripts

| | MRP1 | cMOAT | MRP3 | MRP4 | MRP5 |
|-----------------|------|-------|------|------|------|
| Lung | •••• | ○ | • | • | •• |
| Kidney | ••• | • | • | • | •• |
| Bladder | •••• | ○ | • | • | •• |
| Spleen | •••• | ○ | • | ○ | •• |
| Mamma | nd | ○ | ○ | nd | nd |
| Salivary gland | nd | ○ | ○ | ○ | •• |
| Thyroid | •••• | ○ | ○ | ○ | • |
| Testis | •••• | ○ | ○ | ○ | •• |
| Nerve | • | • | ○ | ○ | •• |
| Stomach | ••• | ○ | • | ○ | •• |
| Liver | ○ | •••• | •••• | ○ | • |
| Gall bladder | ••• | nd | nd | • | •• |
| Duodenum | •• | •• | ••• | nd | nd |
| Colon | ••• | ○ | ••• | ○ | •• |
| Adrenal gland | •••• | ○ | ••• | ○ | • |
| Skeletal muscle | •• | ○ | ○ | ○ | •••• |
| Heart | • | ○ | ○ | ○ | •• |
| Brain | • | ○ | ○ | ○ | ••• |
| Placenta | •• | ○ | ○ | ○ | • |
| Ovary | •• | ○ | ○ | ○ | • |
| Pancreas | • | ○ | • | ○ | • |
| Tonsil | nd | ○ | • | • | •• |

nd = not determined

○ = no expression

• • • • • = low to high expression

Table 3

Characteristics of the doxorubicin-selected MDR cell lines analyzed

| Cell line | Source | Drug used for selection | RNA levels | | | | | | |
|-----------------------|----------------------------|-------------------------|------------|------|-------|------|------|------|--|
| | | | MDR1 | MRP1 | cMOAT | MRP3 | MRP4 | MRP5 | |
| S1 | Non small cell lung cancer | | ⊕ | • | ⊕ | ⊕ | • | • | |
| 30.3M | | | ⊕ | •• | ••• | ••• | • | • | |
| 1R50b | | dox (30 nM) | ○ | • | •• | • | • | • | |
| 2R50 | | dox (50 nM) | ○ | •• | ⊕ | ⊕ | • | • | |
| 3R80 | | dox (80 nM) | ○ | • | ⊕ | •• | • | • | |
| 2R120 | | dox (120 nM) | • | •• | •• | •• | • | • | |
| 2R160 | | dox (160 nM) | •••• | •• | ••• | • | • | • | |
| 1R500 | | dox (500 nM) | •••• | • | •• | ⊕ | • | • | |
| COR-L23 | Non small cell lung cancer | | ○ | • | ○ | ○ | • | ⊕ | |
| COR-L23/R | | dox (368 nM) | ○ | •••• | ○ | ○ | • | ⊕ | |
| GLC ₄ | Small cell lung cancer | | ○ | • | ○ | ○ | • | • | |
| GLC ₄ /ADR | | dox (1160 nM) | ○ | •••• | ○ | ○ | • | • | |
| MOR/P | Adeno lung carcinoma | | ○ | • | •••• | ••• | • | ••• | |
| MOR/R | | dox (368 nM) | ○ | •••• | •••• | ••• | • | ••• | |
| HL60 | Leukemia carcinoma | | ○ | • | ○ | ○ | • | ⊕ | |
| HL60/ADR | | dox (186 nM) | ○ | •••• | ○ | ○ | • | ⊕ | |

○ = no expression

⊕ = very low expression

• • • • • = low to high expression

Table 4

Characteristics of the cell lines selected for resistance to cisplatin, tetraplatin or CdCl₂

| Cell line | Source | Drug used for selection | RNA levels | | | | | | GSH (nmol/mg protein) |
|---------------|----------------------|---|------------|------|-------|------|------|------|-----------------------|
| | | | MDR1 | MRP1 | cMOAT | MRP3 | MRP4 | MRP5 | |
| T24 | Bladder carcinoma | | ○ | ● | ⊕ | ⊕ | ● | ● | 11.4 ± 2.4 |
| T24/DDP5 | | CDDP (3.3 μM) | ● | ● | ⊕ | ⊕ | ● | ● | 26.8 ± 0.1 |
| T24/DDP7 | | CDDP (4.7 μM) | ● | ● | ⊕ | ⊕ | ● | ● | 65.5 ± 1.8 |
| T24/DDP10 | | CDDP (6.7 μM) | ● | ● | ⊕ | ⊕ | ● | ● | 59.5 ± 3.3 |
| 2008 | Ovarium carcinoma | | ● | ● | ⊕ | ⊕ | ⊕ | ● | 33.5 ± 1.5 |
| 2008/C13*5.25 | | CDDP (5.0 μM) | ○ | ● | ● | ⊕ | ⊕ | ● | 113.2 ± 16.6 |
| 2008/MT | | CdCl ₂ (25 μM)/ ZnCl ₂ (200 μM) | ● | ● | ⊕ | ⊕ | ⊕ | ● | 48.5 ± 5.4 |
| 2008/A | | CDDP (0.5 μM) | ○ | ● | ● | ⊕ | ⊕ | ● | 124.7 ± 18.6 |
| A2780 | Ovarium carcinoma | | ○ | ● | ⊕ | ○ | ⊕ | ● | 13.3 ± 0.9 |
| A2780/DDP | | CDDP (50.0 μM) | ○ | ● | ● | ○ | ⊕ | ● | 26.5 ± 2.4 |
| PXN94 | Ovarium carcinoma | | ⊕ | ● | ○ | ○ | ● | ● | 40.2 ± 5.6 |
| PXN94/teR | | Tetraplatin (2.0 μM) | ⊕ | ● | ○ | ○ | ● | ● | 91.8 ± 8.8 |
| HCT8 | Colon carcinoma | | ● | ● | ● | ● | ● | ● | 17.7 ± 2.2 |
| HCT8/DDP | | CDDP (50.0 μM) | ● | ● | ● | ● | ● | ● | 70.2 ± 6.8 |
| GCT27 | Testis carcinoma | | ○ | ● | ⊕ | ○ | ⊕ | ● | 5.2 ± 0.2 |
| GCT27/cisR | | CDDP (4.0 μM) | ○ | ● | ⊕ | ○ | ⊕ | ● | 9.2 ± 3.5 |
| KB-3-1 | Epidermoid carcinoma | | ○ | ● | ● | ● | ⊕ | ● | 36.7 ± 0.9 |
| KCP-4(-) | | CDDP (23.3 μM) | ○ | ● | ● | ● | ⊕ | ● | 72.7 ± 8.9 |
| KCP-4(+) | | CDDP (6.7 μM) | nd | nd | ⊕ | nd | nd | nd | 158.4 ± 13.3 |

○ = no expression

⊕ = very low expression

● = low to high expression

nd = not determined

Table 5

| Cell line | Cisplatin | | Doxorubicin | | RNA levels | |
|---------------|-----------------------|------|-----------------------|-----|------------|-------|
| | IC ₅₀ (nM) | RF | IC ₅₀ (nM) | RF | MRP1 | cMOAT |
| S1 | 144 | - | 13.8 | - | • | ⊖ |
| 30.3M | 500 | 3.5 | 51.1 | 3.7 | •• | •••• |
| 1R50b | 194 | 1.5 | 84.2 | 6.1 | • | •• |
| 2R50 | 115 | 0.9 | 69.0 | 5.0 | •• | ⊕ |
| 3R80 | nd | nd | 75.9 | 5.5 | • | ⊕ |
| 2R120 | 313 | 2.2 | 345 | 25 | •• | •• |
| 2R160 | 600 | 4.2 | 1380 | 100 | •• | •••• |
| 1R50C | 260 | 1.7 | 3450 | 250 | • | •• |
| T24 | 825 | - | 6.5 | - | • | ⊖ |
| T24/DDP5 | 2200 | 2.7 | 5.5 | 0.8 | • | ⊖ |
| T24/DDP7 | 1800 | 2.2 | 3.0 | 0.5 | •• | ⊖ |
| T24/DDP10 | 8000 | 9.7 | 6.5 | 1.0 | •• | ⊖ |
| 2008 | 340 | - | 57.6 | - | •• | ⊕ |
| 2008/C13*5.25 | 3000 | 8.8 | 43.2 | 0.8 | •• | • |
| 2008/MT | 210 | 0.6 | 54.4 | 0.9 | •• | ⊕ |
| 2008/A | 680 | 2.0 | 36.8 | 0.6 | •• | • |
| A2780 | 430 | - | 5.2 | - | • | ⊕ |
| A2780/DDP | 4300 | 10.0 | 6.2 | 1.2 | • | •••• |
| PXN94 | 580 | - | 4.2 | - | •• | • |
| PXN94/tetR | 2400 | 4.1 | 11.0 | 2.6 | •• | • |
| HCT8 | 1900 | - | 90.0 | - | • | • |
| HCT8/DDP | 4800 | 2.5 | 95.0 | 1.1 | •• | •••• |
| GCT27 | 288 | - | 3.0 | - | • | ⊕ |
| GCT27cisR | 2100 | 7.3 | 3.1 | 1.0 | • | ⊕ |
| KB-3-1 | 380 | - | 11.0 | - | •• | •••• |
| KCP-4(-) | 700 | 1.8 | 43.0 | 3.9 | •• | •••• |
| KCP-4(+) | 22400 | 58.9 | 8.0 | 0.7 | nd | ⊕ |

CLAIMS

1. A nucleic acid comprising a sequence encoding at least a part of a member of a family of organic anion transporters, with the exclusion of mammalian Multidrug Resistance Associated Protein, said nucleic acid comprising
5 at least a gene family specific fragment of one of the sequences of fig.1a or fig.1b or the complement thereof, or a sequence having at least 55%, preferably 70%, in particular 90% homology therewith.
2. A nucleic acid according to claim 1 encoding at least a
10 part of a mammalian member of said family.
3. A nucleic acid according to claim 2 encoding at least a part of a human member of said family.
4. A nucleic acid and/or its complement having at least part of the sequence of fig.1A and encoding a protein having
15 human Canalicular Multispecific Organic Anion Transport protein or similar activity or antigenicity.
5. A nucleic acid and/or its complement having at least part of the sequence of fig.1b and encoding a protein having rat Canalicular Multispecific Organic Anion Transport
20 protein activity or antigenicity.
6. A nucleic acid encoding rat Canalicular Multispecific Organic Anion Transport protein or human Canalicular Multispecific Organic Anion Transport protein.
7. A vector comprising a nucleic acid according to anyone
25 of the foregoing claims and suitable means for replication, transduction and/or expression of said nucleic acid.
8. A vector according to claim 7 further comprising a gene encoding a therapeutically beneficial protein.
9. A vector according to claim 7 or 8 further comprising a
30 gene encoding glutathion elevating activity.
10. A vector according to claim 9 wherein the gene encodes at least a functional part of a gamma glutamyl cysteine synthetase or a UDP-glucuronosyltransferase.

11. A vector according to claim 7 wherein the therapeutically beneficial protein is another multidrugresistance related protein such as MDR1.
12. A cell comprising a nucleic acid or a vector according to anyone of the foregoing claims.
13. A cell according to claim 12 further comprising a vector encoding glutathion elevating activity.
14. A cell according to claim 12 or 13 provided with other resistance related proteins such as MDR1.
15. A method for providing cells with Canalicular Multispecific Organic Anion Transport protein activity, comprising transducing said cell with a nucleic acid or a vector according to anyone of claims 1-11.
16. A method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to claim 15, comprising increasing the intracellular level of glutathion, glucuronide and/or sulphate.
17. A method for enhancing Canalicular Multispecific Organic Anion Transport protein activity of cells according to claim 15, comprising enhancing the conjugating capacity and/or the compelxing activity of said cell for sulphate, glutathion, glucuronide and the like.
18. A method for reducing Canalicular Multispecific Organic Anion Transport protein activity and/or the multidrug resistance of a cell comprising providing said cell with an antisense construct of a nucleic acid or a vector according to anyone of claims 1-11.
19. A method according to claim 18, further comprising providing the cell with an antisense construct derived from another multidrug resistance related protein such as MDR1.
20. A protein encoded by a nucleic acid according to anyone of claims 1-6 or obtainable by expression of a vector according to anyone of claims 7-11.
21. A protein having Canalicular Multispecific Organic Anion Transport protein activity or Canalicular Multispecific Organic Anion Transport protein specific

antigenicity comprising at least part of the sequence of fig.4 or another mammalian equivalent thereof.

22. Use of a nucleic acid according to anyone of claims 1-6 or a protein according to claim 20 or 21 in the diagnosis of
- 5 Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein.
23. Use of a nucleic acid according to anyone of claims 1-6 or a protein according to claim 20 or 21 in the treatment of
- 10 Dubin-Johnson disease, Rotor disease or another disease involving Canalicular Multispecific Organic Anion Transport protein.
24. Use of a nucleic acid according to anyone of claims 1-6 as a selectable marker gene.

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1 AGGATAATTC CTGTTCCACT TTCTTTGATG AAACAAGTAA AGAAGAAACA
51 ACACAATCAT ATTAATAgAA GAGTctTCgt TCCAGACgCA GtCCAGGAAT
101 CATGCTGGAG AAgTTCTGCA ACTctACTTTT tTGGAAATTcC TCATTcCtTgg
151 ACAGTCCGGA GGCAGAcCTG cCacTTTGTt TtGAGCAAac TGTTctTGGTG
201 TGGATTCCcT TGGGCTTCcT ATGGCTCCTG GCCCCctGgC agcTTctCCa
251 cGtGtAtAAA tCCaggACCA AGaGATCcTc TACCACCAAA cTctatctTG
301 cTAaGCaGGT ATTcGttGgT tTtCttctTA TtctAgCagc CATAGAGCtG
351 GCCCTTGTAC TCACAGaAGA CtCTGGACAA GCCACAGTCC cTGCTGTtCG
401 ATATACCAAT CCAAGcCTcT ACCTAGgCAC AtGGcTCCTG GTTTTGCTGA
451 TCCAATACaG cAGACAATGG TGTGTACAGA AAAACTCCTG GTtCCTGTcCC
501 cTATTcTGGA TTCTCTcGAT ACTCtGtGGC ACTTTcCAaT TTCAGACTCt
551 gATccGGAcA CTCTTACAGG GTGACAAAtc TAATCTAGCc TACTCcTGcC
601 TGTtctTCAt CtCctAcGGa tTcCaGATCc TGATCCTGAT CtTTTCAGCA
651 TTTTCAGAAA ATAATGAgTC ATCAAATAAT CCATCATCCA TAGCTTCAtT
701 CCTGAgTAgC ATTACCTACA GCTGGTATGA CAgCaTCATT CTGAAAgGcT
751 ACaAgCgTcC TCTGACACTc gAgGATgTcT GGGAAgttGA TGAAgAgATg
801 AAAACCaAGA CATTAGTGAG CAAGTTTGaa ACGCACATGA AGAGAGAGCT
851 GCAGAAAGCC AGGCGGGCAC TCCAGAGACG GCAGGAGAAG AGCTCCCAGC
901 AGAACTCTGG AgCCAGGCTG CCTGGCTTGA ACAAGAATCA GAGTCAAAGC
951 CAAGATGCCC TTGTCTGGA AGATGTTGAA AAGAAAAAAA AGAAgTCTGG
1001 GACCAAAAAA GATgTTCCAA AATCCTGGTT GATGAAgGct CTGTTCAAAA
1051 CTTTCTACAT GGTGCTCCTG AAATCaTTCC TACTGAAgCT AGTGAATGAC
1101 aTCTTCACgT TTGTGAGTCC TCAGCTGCTG AAAtTGCTGA TCTCctTTGC
1151 AAGTGAcCGt GACaCATAtT TGTGGATTGG ATATCTCTGT GcAtCCTCT
1201 TAtTCaCTGC GgCtCTcATT CAGTctTTct GCCTTCAgTG TtAtTTCCAA
1251 CTGTGCTTCA AGCTGGGTGT AAAAgTACGg ACAGCtATCa tgGcTTctGT
1301 ATATAAGAAG GCATTGACCC TAtCCAACTT GGCCaGGAAG GAGTACACCG
1351 tTGGAGAAAC AGTGAACCTG ATGTCTGTGG ATGCCCAGAA GcTCATGGAT
1401 GTGACCAACT TCATGCACAT GtGTGGTCA AGTGTTctAC AGATTGTCTT

FIG. 1a

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1451 ATCTATCTTC TTCCTAtGGA GAGAGTTGGG ACCCTCAGTC TTAGCAGGTG
1501 TTGGGGTGAT GGTGCTTGTA ATCCCAATTA ATGCGATACT GTCCACCAAG
1551 AGTAAGACCA TTCAGGTCAA AAATATGaAG AATAAAGACA AACGTTTAAA
1601 GATCATGAAT GAGATTCTTA GTGGAATCAA GATCCTGAAA TATTTTGCCT
1651 GGGAACTTC ATTCAGAGAC CAAGTACAAA ACCTCCGGAA GAAAGAGCTC
1701 AAGAACCTGC TGGCCTTTAG TCAAcTACAG TGTGTaGTAA TATTcGTCTT
1751 CCAGTTAACT CCAGTcCTGG TATCTGTGGT CACATTTTCT GTTTATGTCC
1801 TGGTGGATaG CAACAATATT TcGGATGCAC AAAAGGCCTT CACCTCCATT
1851 ACCCTCTTCA ATATCCTGCG CTTCCCCCTG AGCATGCTTC CCATGATGAT
1901 CTCCTCCATG CTCCAGGCCA GTGTTTCCAC AGAGCGGCTA GAGAAGTACT
1951 TGGGAGGGGA/TGACTTGGAC ACATCTGCCA TTCGACATGA CTGCAATTTT
2001 GACAAAGCCA TGCAGTTTTC TGAGGCCTCC TTTACCTGGG AACATGATTC
2051 GGAAGCCACA GTCCGAGATG TGAACCTGGA CATTATGGCA GGCCAACTTG
2101 TGGCTGTGAT AGGCCCTGTC GGCTCTGGGA AATCCTCCTT GATATCAGCC
2151 ATGCTGGGAG AAATGGAAAA TGTCCACGGG CACATCACCA TCAAGGGCAC
2201 CACTGCCTAT GTCCACAGC AGTCCTGGAT TCAGAAATGGC ACCATAAAGG
2251 ACAACATCCT TTTTGGAAAC GAGTTTAAATG AAAAGAGGTA CCAGCAAGTA
2301 CTGGAGGCCT GTGCTCTCCT CCCAGACTTG GAAATGCTGC CTGGAGGAGA
2351 TTTGGCTGAG ATTGGAGAGA AGGGTATAAA TCTTAGTGGG GGTCAGAAGC
2401 AGCGGATCAG CCTGGCCAGA GCTACCTACC AAAATTTAGA CATCTATCTT
2451 CTAGATGACC CCCTGTCTGC AGTGGATGCT CATGTAGGAA AACATATTTT
2501 TAATAAGGTC TTGGGCCCCA ATGGCCTGTT GAAAGGCAAG ACTCGACTCT
2551 TGGTTACACA TAGCATGCAC TTTCTTCCTC AAGTGGATGA GATTGTAGTT
2601 CTGGGGAATG GAACAATTGT AGAGAAAGGA TCCTACAGTG CTCTCCTGGC
2651 CAAAAAAGGA GAGTTTGCTA AGAATCTGAA GACATTTCTA AGACATACAG
2701 GCCCTGAAGA GGAAGCCACA GTCCATGATG GCAGTGAAGA AGAAGACGAT
2751 GACTATGGGC TGATATCCAG TGTGGAAGAG ATCCCCGAAG ATGCAGCCTC
2801 CATAACCATG AGAAGAGAGA ACAGCTTTTCG TCGAACACTT AGCCGCAGTT
2851 CTAGGTCCAA TGGCAGGCAT CTGAAGTCCC TGAGAAACTC CTTGAAAACCT
2901 CGGAATGTGA ATAGCCTGAA GGAAGACGAA GAACTAGTGA AAGGACAAAA
2951 ACTAATTAAG AAGGAATTCA TAGAACTGG AAAGGTGAAG TTCTCCATCT
3001 ACCTGGAGTA CCTACAAGCA ATAGGATTGT TTTGATATT CTTCATCATC

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3051 CTTGCGTTTG TGATGAATTC TGTGGCTTTT ATTGGATCCA ACCTCTGGCT
3101 CAGTGCTTGG ACCAGTGA CTAAATCTT CAATAGCACC GACTATCCAG
3151 CATCTCAGAG GGACATGAGA GTTGGAGTCT ACGGAGCTCT GGGATTAGCC
3201 CAAGGTATAT TTGTGTTTAT AGCACATTTT TGGAGTGCCT TTGGTTTCGT
3251 CCATGCATCA AATATCTTGC ACAAGCAACT GCTGAACAAT ATCCTTCGAG
3301 CACCTATGAG ATTTTTTGAC ACAACACCCA CAGGCCGGAT TGTGAACAGG
3351 TTTGCCGGCG ATATTTCCAC AGTGGATGAC ACCCTGCCTC AGTCCTTGCG
3401 CAGCTGGATT ACATGCTTCC TGGGGATAAT CAGCACCTT GTCATGATCT
3451 GCATGGCCAC tCCTGTCTTC ACcATcATCG TcATTCTCT tGGcAttAtt
3501 tAtGtATctG TtCAGAtGtT ttAtGtGTct ACCTCCCGCC AGctGAGGcG
3551 TCTGGACTCT GTCACCAGgT CCCCAATcTA cTcTCAcTTC AGCGAGACCG
3601 TATCAGGTTT GCCAGTTATC cGTGCCTtTG AGCACCAGCA GcGATTtTG
3651 AAACACAATG AGGAGAGGat TGACACCaAC CAGAAAtGTG TcTTTTccTG
3701 GATCACCTCC AACAGGtGGc TTGCAATTcG CCTGGAGcTG GTTGGGAACC
3751 TGACTGTcTT cTTTTcAGCC tTGaTGATGG TTATTtAtAG aGATACcCTA
3801 AGtGGGGACA CTGTTGGcTT TGTtTGTC AATGCActCA ATATCACACA
3851 AACCCTGAAC TGGcTGGtGa GGatGACaTC aGaAaTaGaG aCCaACATTG
3901 tGGcTGTtGA GCGaATAAct GAGtACACAA AAGTGGAAaA TGAGGCACCC
3951 TGgGTGActG ATAAGAgGcc tCCGCCAGAT TGGcCCAGCA AAGGCAAGAT
4001 CCAGtTTAAC AAcTaCCAAG TGGGTACCG ACCTGAgCTG GATCTGGTCC
4051 tCaGAgGGAT CACTTGTGAC ATCgGTAgCA TGGAgAAgAt TGGTGTGGTG
4101 GGCagGAcAG gAgctGGAAa gTcatcCCTC ACAAactGcC TcTTcaGaAT
4151 cTTaGaGGct GCCGtGGTC aGATTatcAT TGATGGaGTA gAtATTGcTT
4201 cCATTgGGcT CCACGAcCTc CGAGaGAAGc TGAcCaTcAT cCCCCAGGAc
4251 CCCATcCTGT TcTcTGGaAG cCTGAGGaTG aATcTcGACC cTTTCAACAA
4301 cTAcTCaGAT GAGGAgATTT GGAAGGCCTT GGAGcTGGcT CACcTCAAgT
4351 CTTTTGTGGC CAGcCTGCAA CTGgGGTTAT CCCACGAAGT taCAGAgGCT
4401 gGTgGCAACC TGAGCATAgG CCAGAggcag CTGCTGTGcC TGGGCAGGcC
4451 TcTgcTTcGG AAATCCAAGA TCCTGGTCCT GGATGAgGCC ACTGCTGCGG
4501 TGGATCTAGA GACAGACaAc CTCATTcAGA CGACCATCCA AAACGAgTTC
4551 GCCCACTGCA CAGTGATCAC CATCGCCAC AGGCTGCATA CCATCATGGA
4601 CAGTGaCaAg GTaATGGtCC tAGaCaAcgG GaAgaTtAtA gAgTACGGCa

4651 gCCcTGaAGA acTgCtaCAA ATCCCTGGAc CCTTTtACtT TATGGCTAAG
4701 GaAgCTgGCA TTGAgAATgT gAaCagCaCa aAAtTcTagc agaaggcCCC
4751 ATgGGtTaga AAagGactat AagaatAATT tCtTAtttAa ttttAttttt
4801 tataaaAAtaC aGaataCata CaaaagtgTg taTaaAATGt ACgTTTTaaa
4851 aaAGGaTAag TgaacACCCa TGAACctact ACCcAgGTTA AgaaaaataAa
4901 tgTCaCCAGg TactTGAgAA ACCcctcgAt TGTctACcTC gATCgTactT
4951 CctTGcTACC caCCCctcCC AGGgacAAcC AcTgTCcTGA attTcaCgAt
5001 AATtattCCt tTGCCTtTca tTTCTGTTTT ATCACCTTTG TATGTATCTT
5051 TAAACAACAT ATACCCTTTT TtACTTATGT AAATGGACTG ACTCATACTG
5101 CAtACAtcTT cTATGAcTTG AtTcTTTTGT tCAAtAttat AtctGagatT
5151 cAtCCATGGT GATGCAAATA GGTGCATTAt TTTTTTTCAC TGCTCTGTag
5201 TCTGGCattg tatGaATacA gcacaatgtA tcagTtttaa Tattggggat
5251 catTagcatt atTctcaggt tttTaaaaAt tAtaagcAgt actactatgg
5301 AaaGAAAnTC TGGTCCTACA TCTCCCTGGC ACATATGTAA GAGTTTCnCh
5351 AGGGTAtAAC CtAGGAATGG AGGGTATGAA CATGTTTACA TGCACAAACT
5401 AGctGATGCC AAActGGTAA TGCCAACTGA AAACAtTGCT GTCAAtCTGA
5451 TGAATATGAA TTGATGTATC AATGAGAATT TCATTtGCAT TTCCCTAGTA
5501 TCTATTGGGG ATGAATATAT TTTCATGtTT CTGGGGCATT TGCATTtCCA
5551 CTTGTTTTTT aAATATTGTG TCTGATAwTT yTnnAT

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1 TGCAC TTTAA CATCTGCTTT CCCAGAGGAA AAAGTAAAGG AGAAACAGTA
51 CAATCATAGA AGAGTCTTCG TAACAGAAGC GCGAGGAGAG CATTATGGAC
101 AAGTTCTGCA ACTCTACTTT TTGGGATCTC TCATTACTGG AAAGTCCAGA
151 GGCTGACCCG CCTCTTTGTT TTGAGCAAAC TGTTCCTGGTG TGGATTCCCT
201 TGGGCTTTCT TTGGCTCCTG GCTCCTTGGC AACTTTACAG CGTGACAGTA
251 TCCAGGACCA AGAGATCTTC TATAACCAA TTCTACCTTG CCAAGCAGGT
301 GTTCGTCGTG TTTCTTCTTA TTTTAGCAGC CATAGACCTG TCTCTTGCGC
351 TCACAGAAGA TACTGGACAA GCCACAGTTC CTCCTGTCAG ATATACGAAT
401 CCAATCCTCT ACCTGTGCAC ATGGCTCCTG GTTTTGGCAG TCCAGCACAG
451 CAGGCAATGG TGTGTACGAA AGAACTCTTG GTTCCTGTCT CTGTTCTGGA
501 TCCTCTCGGT CTTATGCGGC GTATTCCAGT TTCAGACTCT GATACGAGCA
551 CTCCTGAAGG ACAGCAAGTC CAACATGGCC TACTCCTACC TGTTCCTCGT
601 CTCCTACGGT TTCCAGATTG TCCTCCTGAT TCTTACAGCC TTTTCAGGAC
651 CAAGTGACTC AACACAAACT CCATCAGTCA CGGCTTCCTT TCTGAGTAGC
701 ATTACATTTA GTTGGTATGA CAGGACTGTT CTGAAAGGTT ACAAGCATCC
751 ACTGACACTA GAAGATGTCT GGGATATCGA TGAAGGGTTT AAAACAAGGT
801 CAGTCACCAG CAAGTTTGAG GCGGCCATGA CAAAGGACCT GCAGAAAGCC
851 AGGCAGGCTT TTCAGAGGCG GCTGCAGAAG TCCCAGCGGA AACCTGAGGC
901 CACACTACAC GGA CTGAACA AGAAGCAGAG TCAGAGCCAA GACGTTCTCG
951 TCCTGGAAGA AGCGAAAAAG AAGTCTGAGA AGACCACCAA AGACTATCCC
1001 AAATCGTGGT TGATCAAGTC TCTCTTCAA ACCTTCCACG TAGTGATCCT
1051 GAAATCATTT AACTGAAAT TAATACATGA CCTTTTGGTG TTTCTGAATC
1101 CTCAGCTGCT GAAGTTGCTG ATCGGTTTCG TGAAGAGCTC TAACTCATAC
1151 GTGTGGTTTG GCTATATCTG TGCAATCCTA ATGTTTGCTG TGA CTCTCAT
1201 CCAATCTTTC TGCCTTCAGT CTTACTTTCA ACATTGTTTT GTGTTGGGAA
1251 TGTGCGTACG GACAACCGTC ATGTCTTCGA TATATAAGAA GGCATTGACC
1301 CTATCTAACT TGGCTAGGAA GCAGTACACC ATTGGAGAGA CGGTGAACTT
1351 GATGTCTGTA GATCCCAGA AGCTAATGGA TGCGACCAAC TACATGCAGT
1401 TGGTGTGGTC AAGTGTTATA CAGATTACTT TGTCCATCTT CTTCTGTGG

FIG. 1b

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1451 AGAGAGTTGG GACCGTCCAT CTTAGCAGGT GTTGGGGTTA TGGTTCTCCT
1501 AATCCCAGTT AATGGAGTTC TGGCTACCAA GATCAGAAAT ATTCAGGTCC
1551 AAAATATGAA GAATAAAGAC AAACGTTTAA AAATCATGAA TGAGATTCTC
1601 AGTGGAATCA AGATCCTGAA ATACTTTGCC TGGGAACCTT CATTTCAAGA
1651 GCAAGTCCAG GGCATTCCGA AGAAAGAACT CAAGAAGTTG CTGCGGTTTCG
1701 GCCAGCTGCA GAGTCTGCTG ATCTTCATTT TACAGATAAC TCCAATCCTG
1751 GTGTCTGTGG TCACATTTTC TGTCTATGTC CTGGTGGATA GCGCCAATGT
1801 TTTGAATGCG GAGAAGGCAT TTACCTCCAT CACCCTCTTC AATATCCTAC
1851 GCTTCCCTCT GTCCATGCTT CCCATGGTGA CCTCATCGAT CCTCCAGGCC
1901 AGTGTTTCTG TGGACCGGCT GGAGAGGTAT TTGGGAGGAG ACGATTTAGA
1951 CACATCTGCC ATTCGCGCCG TCAGCAATTT TGATAAAGCT GTGAAGTTTT
2001 CAGAGGCCTC TTTTACTTGG GACCCGGACT TGGGAAGCCAC AATCCAAGAT
2051 GTGAACCTGG ACATAAAGCC AGGCCAACTG GTGGCTGTGG TGGGCACTGT
2101 AGGCTCTGGG AAATCCTCTT TGGTATCAGC CATGCTGGGA GAAATGGAAA
2151 ACGTTCACGG GCACATCACC ATCCAGGGAT CCACAGCCTA TGTCCCTCAG
2201 CAGTCCTGGA TTCAGAATGG AACCATCAAA GACAACATCC TGTTTGGGTC
2251 CGAATACAAT GAAAAGAAGT ACCAGCAAGT TCTCAAAGCA TGCCTCTCTC
2301 TCCCAGACTT GGAAATATTG CCTGGAGGAG ACATGGCTGA GATCGGAGAG
2351 AAGGGGATAA ATCTCAGTGG TGGTCAGAAG CAGCGAGTCA GCCTGGCCAG
2401 AGCTGCCTAT CAAGATGCTG ACATCTATAT TCTGGACGAT CCCCTGTCCG
2451 CTGTGGATGC TCATGTGGGA AAACACATTT TCAACAAGGT TGTGGGCCCC
2501 AACGGCCTGT TGGCTGGCAA GACGAGAATC TTTGTTACTC ATGGTATTCA
2551 CTTCTTCCC CAAGTGGATG AGATTGTAGT TCTGGGGAAA GGCACCATCT
2601 TAGAGAAAGG ATCCTATCGT GACCTGTTGG ACAAGAAGGG AGTGTTTGCT
2651 AGGAACTGGA AGACCTTCAT GAAGCATTCA GGCCTGAAG GAGAGGCCAC
2701 AGTCAATAAT GACAGTGAGG CGGAAGACGA CGATGATGGG CTGATTCCCA
2751 CCATGGAGGA AATCCCTGAG GATGCAGCTT CCTGGCCAT GAGAAGAGAA
2801 AATAGTCTTC GCCGTACACT GAGCCGCAGC TCTAGGTCCA GCAGCCGACG
2851 TGGGAAGTCC CTCAAAAACCT CCTTGAAGAT TAAAAATGTG AATGTCTTGA
2901 AGGAGAAGGA AAAAGAAGTG GAAGGACAAA AACTAATTAA GAAAGAATTT
2951 GTGGAAACCG GGAAGGTCAA GTTCTCCATC TACCTGAAGT ATCTACAGGC
3001 AGTAGGGTGG TGGTCCATAC TTTTCATCAT CCTTTTCTAC GGATTGAATA

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3051 ATGTTGCTTT TATCGGCTCT AACCTCTGGC TGAGTGCTTG GACCAGTGAC
3101 TCTGACAACT TGAATGGGAC CAACAATTCG TCTTCTCATA GGGACATGAG
3151 AATTGGGGTC TTTGGAGCTC TGGGATTAGC ACAAGGTATA TGTTTGCTTA
3201 TTTCAACTCT GTGGAGCATA TATGCTTGCA GAAATGCATC AAAAGCTTTG
3251 CACGGGCAGC TGTTAACCAA CATCCTCCGG GCACCCATGA GGTTTTTTGA
3301 CACAACTCCC ACAGGCCGGA TTGTGAACAG ATTTTCTGGT GATATTTCTA
3351 CTGTGGACGA CTTGCTCCCC CAGACACTTC GAAGCTGGAT GATGTGTTTC
3401 TTTGGCATCG CTGGCACTCT TGTCATGATC TGCATGGCCA CCCCAGTCTT
3451 CGCTATCATC ATCATTCCTC TCAGCATTCT TTATATTTCTG GTGCAGGTTT
3501 TTTATGTGGC TACTTCCCGC CAGCTGAGAC GGTGAGATTG TGTCACCAAA
3551 TCTCCGATCT ATTCTCACTT CAGTGAGACT GTCACAGGTT TGCCCATTAT
3601 CCGTGCCTTT GAGCACCAGC AGCGATTCTT AGCTTGGAAT GAGAAGCAGA
3651 TTGACATCAA CCAGAAATGT GTCTTTTCCT GGATTACCTC CAACAGGTGG
3701 CTTGCAATTC GGCTGGAGCT GGTTGGAAAC TTGGTCGTCT TCTGTTCCGC
3751 CTTGCTGCTG GTTATTTATA GAAAAACCTT AACCGGGGAC GTTGTGGGCT
3801 TTGTTCTGTC CAACGCCCTC AATATCACAC AAACCTTGAA CTGGCTAGTG
3851 AGGATGACGT CAGAAGCAGA GACCAACATT GTGGCAGTTG AGCGAATAAG
3901 TGAATACATA AATGTAGAGA ATGAGGCGCC CTGGGTGACT GACAAGAGGC
3951 CTCCGGCAGA CTGGCCCAGA CATGGTGAGA TCCAGTTTAA CAACTATCAA
4001 GTGCGGTATC GGCCGGAGCT GGATCTGGTA CTGAAAGGGA TCACTTGTA
4051 CATCAAGAGC GGAGAGAAGG TCGGCGTAGT GGGCAGGACT GGGGCTGGGA
4101 AATCATCCCT CACAACTGC CTCTTCAGAA TCTTAGAGTC TGCGGGGGGC
4151 CAGATCATCA TTGATGGGAT AGATGTTGCC TCCATTGGAC TGCACGACCT
4201 TCGAGAGAGG CTGACCATCA TTCCCCAGGA CCCCATTTTG TTCTCGGGGA
4251 GTCTGAGGAT GAATCTCGAC CCTTTCAACA AATATTCAGA TGAGGAGGTT
4301 TGGAGGGCCC TGGAGTTGGC TCACCTCAGA TCCTTTGTGT CTGGCCTACA
4351 GCTTGGGTTG TTATCCGAAG TGACAGAGGG TGGTGACAAC CTGAGCATAG
4401 GGCAGAGGCA GCTCCTATGC CTGGGCAGGG CTGTGCTTCG AAAATCCAAA
4451 ATCCTGGTCC TGGATGAAGC CACGGCTGCA GTGGATCTGG AGACGGATAG
4501 CCTCATTCAG ACGACCATCC GAAAGGAGTT CTCCCAGTGC ACGGTCATCA
4551 CCATCGCTCA CAGGCTGCAC ACCATCATGG ACAGTGACAA GATAATGGTC
4601 CTAGACAACG GGAAGATTGT CGAGTATGGC AGTCCTGAAG AACTGCTGTC

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4651 CAACAGAGGT TCCTTCTATC TGATGGCCAA GGAAGCCGGC ATTGAAAATG
4701 TGAATCACAC AGAGCTCTAG CAGCTGGTTC CGTGGCTGGC GGACTATAAG
4751 AACAGTTTCT ATTATTTGCT TTGGTTTCTG TGACTGTGCT CTAGGTGCAA
4801 AGACACATAT TTTGTTCCCG TTGCTCAGGC TGGCCTCAAA CTCTAAGGCT
4851 CCAGCAATCT CTGGTCTCAG CCAGAGACCT GTAAAAATAG ACACTTCAAA
4901 GATTATCATG AATAAATA*

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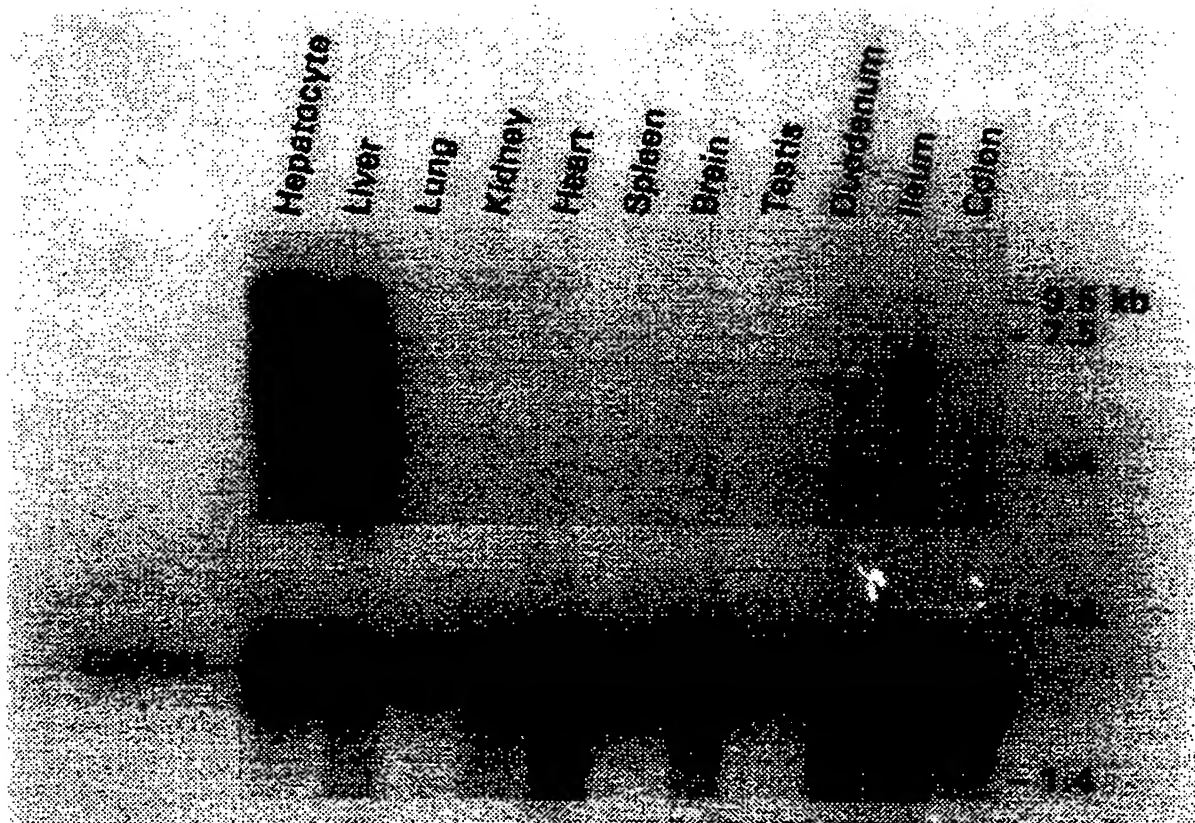


FIG. 2a

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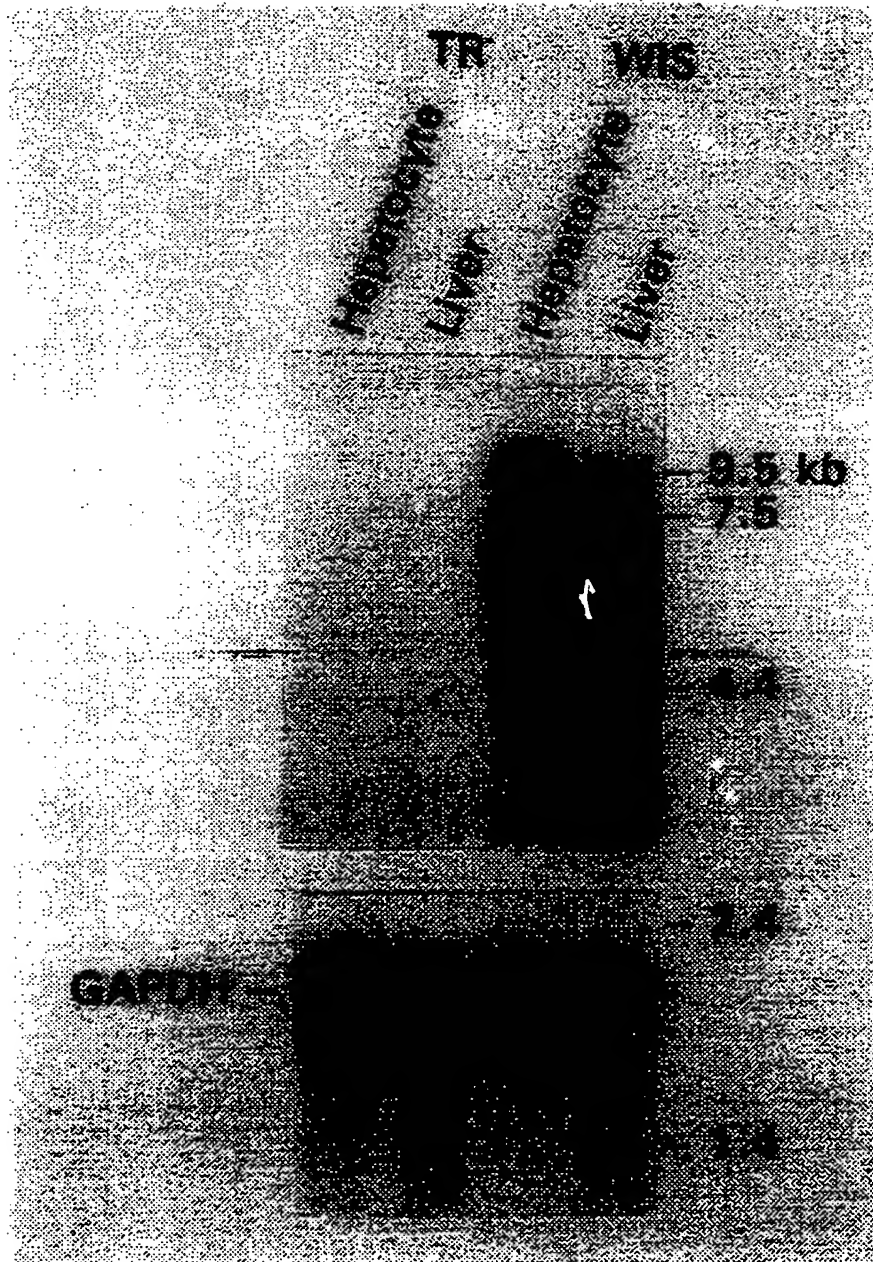


FIG. 2b

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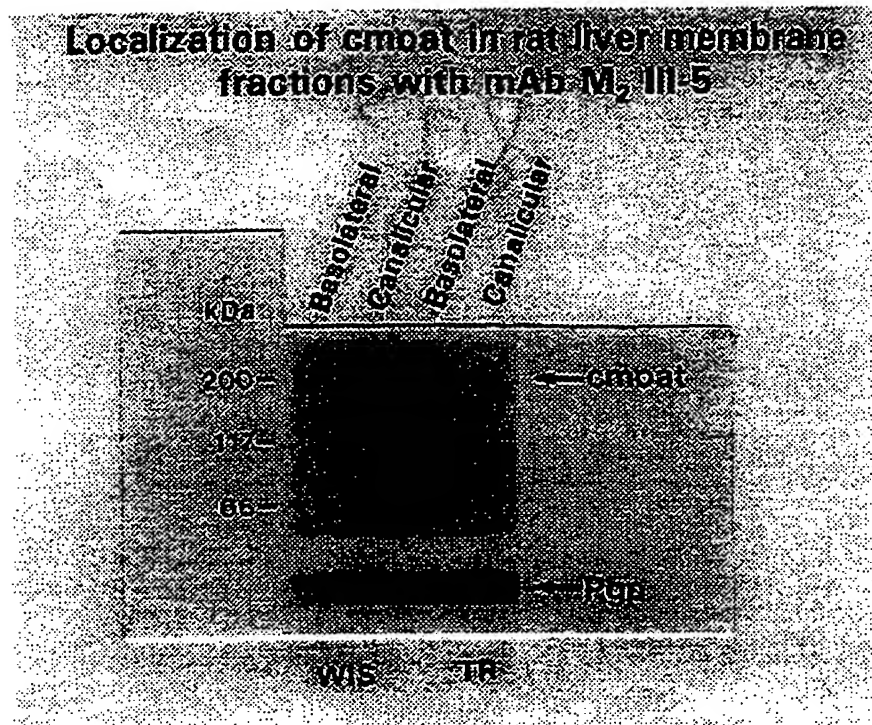


FIG. 3

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1 MDKFCNSTFWDLSSLLESPEADPPLCFEQTVLVWIPLGFLWLLAWPQLYSVYRSRTRKSSI
 61 TKFYLAQVVFVFLILAAIDLSLALTEDTGQATVPPVRYTNPILYLCTWLLVLAVQHSR
 121 QWCVRKNSWFLSLFWILSVLCGVFOFOTLIRALLKDSKSNMAYSYLFFVSYGFQIVLLIL
 181 TAFSGPSDSTQTPSVTASFLSSITFSWYDRTVLKGYKHPLTLEDVWDIDEGFKTRSVTSK
 241 FEAAMTKDLQKARQAFQRRQLQKSQRKPEATLHGLNKKQSQSQDVLVLEEAKKKSEKTTKD
 301 YPKSWLIKSLFKTFHVVILKSFILKLIHDLVFLNPQLLKLLIGFVKSSNSYVWFGYICA
 361 ILMFAVTLIQSPCLQSYFQHCFLGMCVVRTVMSSYKKAITLSNLARKQYTIGETVNLN
 421 SVDSQKLMDATNYMQLVWSSVIQITLSIFFLWRELGPSILAGVGMVLLIPVNGVATKI
 481 RNIQVQNMKNKDKRLKIMNEILSGIKILKYFAWEPFQEQVQGIRKKELKNLLRFGQLQS
 541 LLIFILQITPILVSVVTFSVYVLVDSANVLNAEKAFTSITLFNILRFPLSMLPMVTSSIL
 601 QASVSVDRLERYLGDDLDTSAIRAVSNFDKAVKFSEASFTWDPDLEATIODVNLDIKPG
 661 OLVAVVGTVGSGKSSLVSAMLGEMENVHGHITIOGSTAYVPOOSWIONGTIKDNILFGSE
 ||:::..|||..|||..|||:::..|||..|||..
 rMRP1 GHVTLKGSVAYVPQAWIQNDLSRENILFGRP
 721 YNEKKYQOVLKACALLPDLEILPGGDMAEIGEKGINLSGGOKORVSLARAAAYODADIYIL
 ..|. |..|:..|||..|||..|||:..|||..|||: |
 LQEHCYKAYMEACALLPDLEILPSGDLTEIGEKGVITS rMRP1
 781 DDPLSAVDAHVGKHIFNKVVGPNGLLAGKTRIFVTHGIHFLPQVDEIVVLGKGITILEKGS
 841 YRDLLDKKGVFARNWKTfMKHSGPEGEATVNNDSEAEDDDGLIPTMEEIPEDAASLAMR
 901 RENSLRRTLRSRRSSRRGKSLKNSLKIKNVNLKEKEKEVEGQKLIKKEFVETGKVKF
 961 SIYLKYLOAVGWWSILFIILFYGLNNVAFIGSNLWLSAWTSDSDNLNGTNNSSSHRDMRI
 1021 GVFGALGLAOGICLLISTLWSIYACRNASKALHGQLLTNLRAPMRFFDTPPTGRIVNRF
 1081 SGDISTVDDLLPQTLRSWMMCFFGIAGTLVMICMATPVFAIIIIPLSILYISVQVFYVAT
 1141 SRQLRRLDSVTKSPIYSHFSETVTGLPIIRAFEHQQRFLAWNEKQIDINGKCVFSWITSN
 1201 RWLAIRLEELVGNLVVFCALLLVYRKTLTGDVVGVFLSNALNITQTLNWLVRMTSEAT
 1261 NIVAVERISEYINVENEAPWVTDKRPPADWPRHGEIQFNQVRYRPELDVLKGITCNI
 1321 KSGEKVGVVGRTGAGKSSLTNCLFRILESAGGOIIDGIDVASIGLHDLRERLTIIPODP
 1381 ILFSGSLRMNLDPFNKYSDEEVWRALELAHLRSFVSGLOLGLLSEVTEGGDNLSIGOROL
 1441 LCLGRAVLRSKILVLDEATAAVDLETDLSLIOTTIRKEFSQCTVITIAHRLHTIMDSKI
 1501 MVLNKGKIVEYGSPEELLSNRGSFYLMKEAGIENVNHTL

FIG. 4

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GS-DNP transport in COS-7 cells transfected with cMOAT/'Mock'; T = 15°C

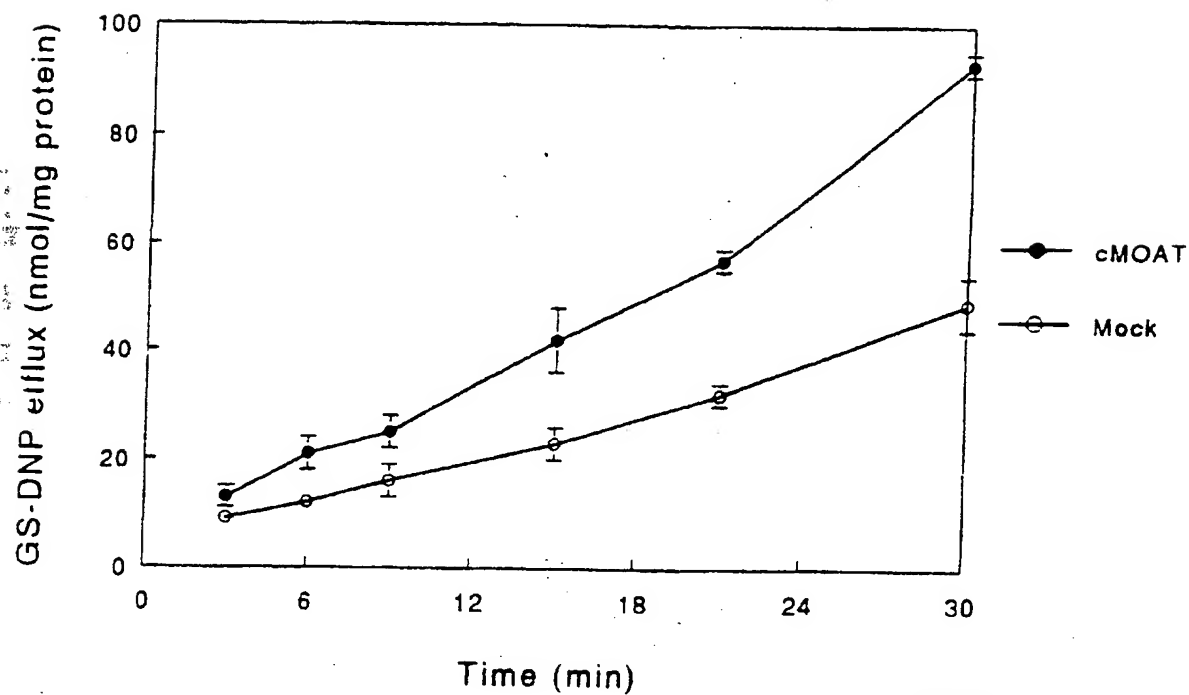


FIG. 5

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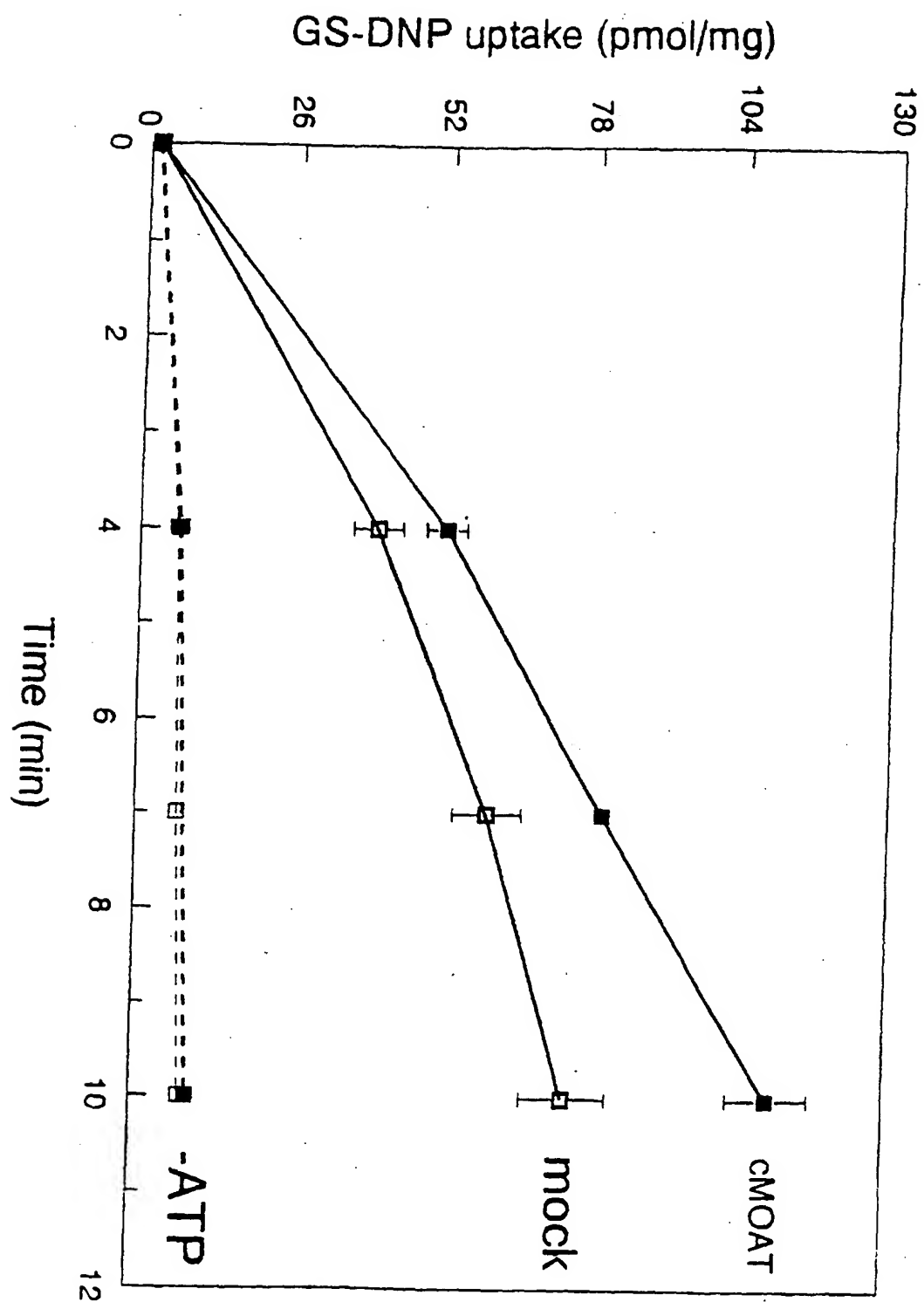


FIG. 6

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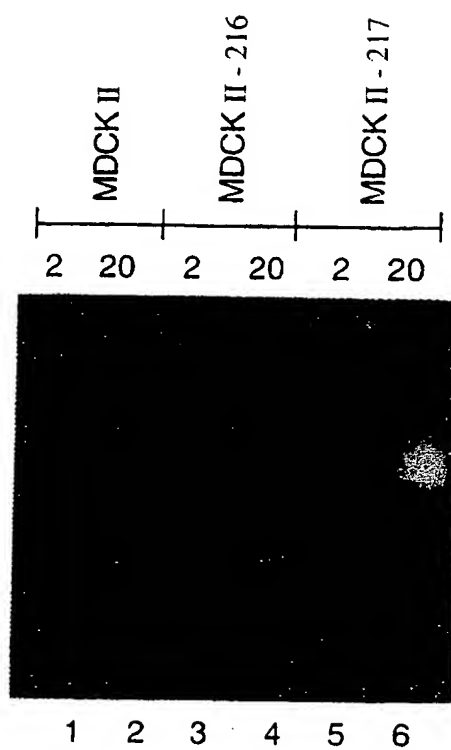


FIG. 7

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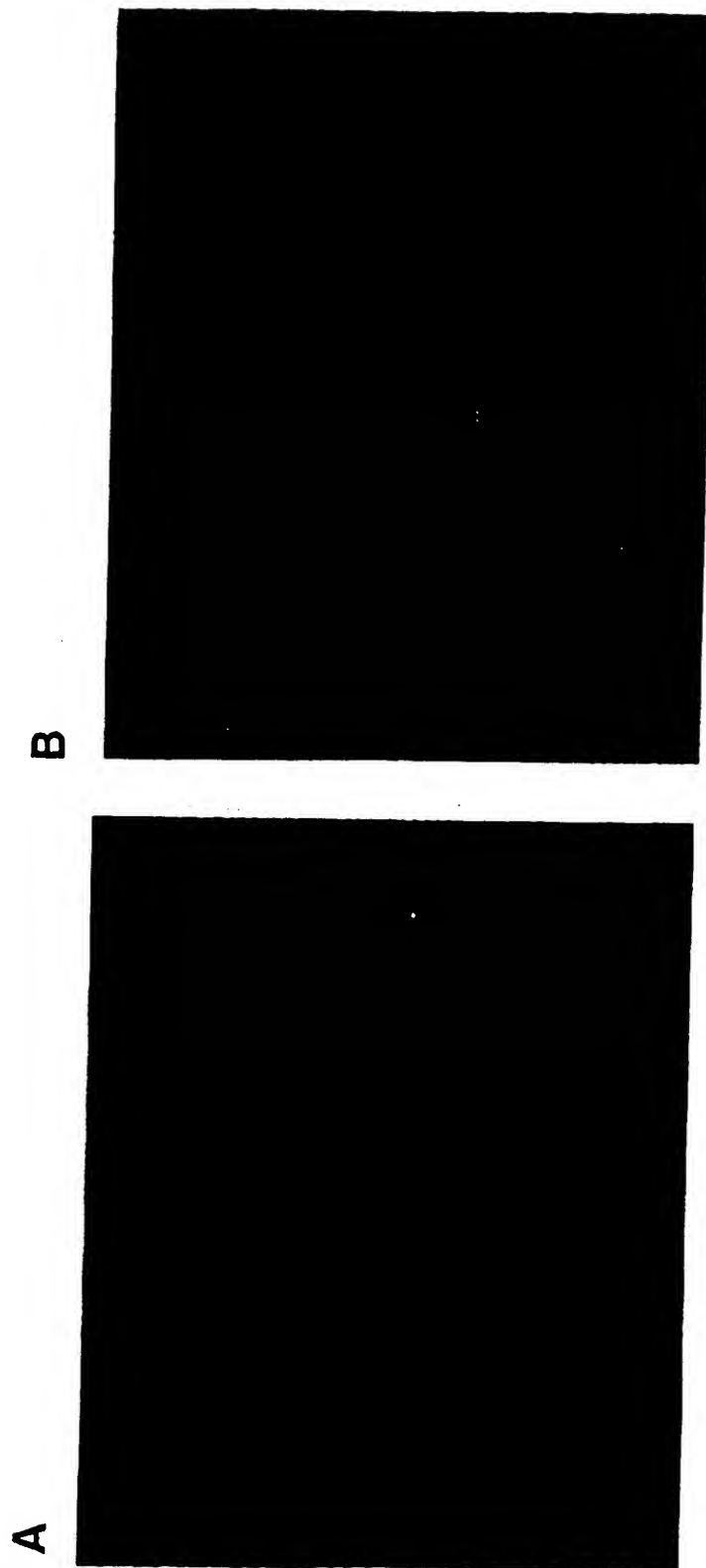


FIG. 8

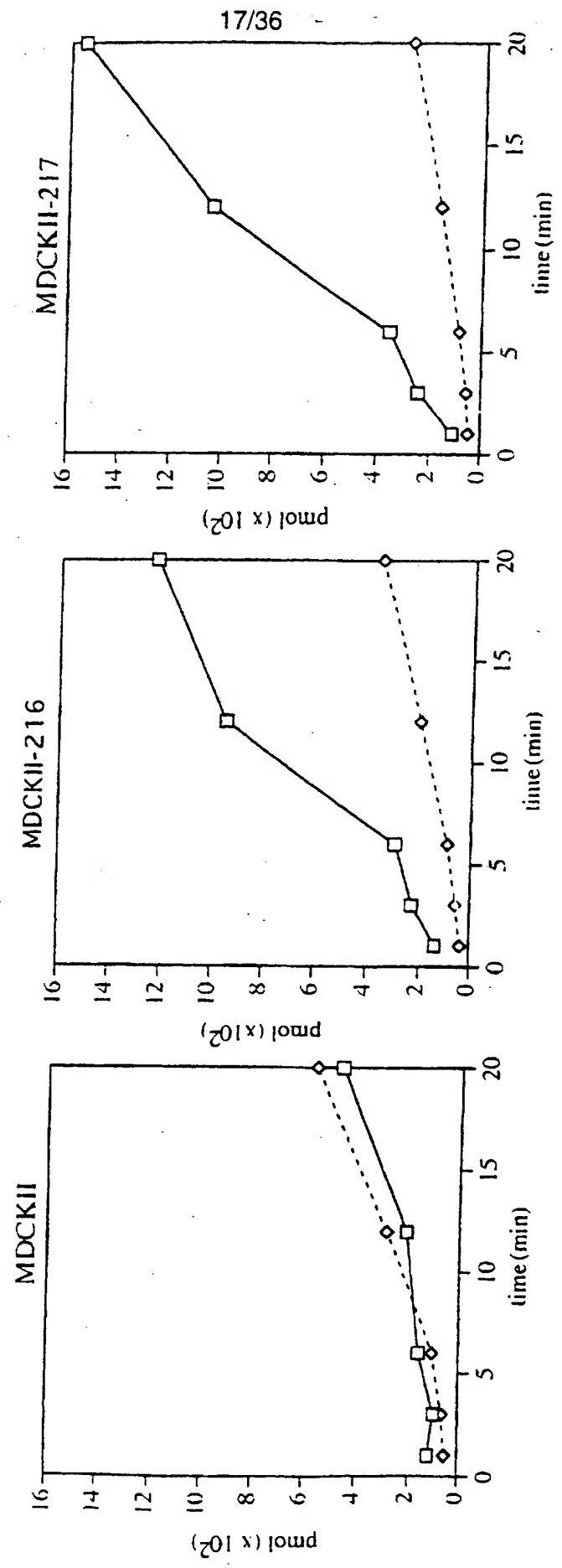


FIG. 9

 1 MLEKFCNSTF WSSSFLOSPE ADLPLCFEOT VLVWIPLGFL VLLAPWQLH VYKSRKRSS TTKLYLAKOV FVGFLILAA IELALVLTED SGOATVPAVR
 101 YTNPSLYLGT WLLVLLIQYS ROWCVOKNSW FLSLFWLSI LCGTFQFOTL IRTLLQGDNS NLAYSCLFFI SYGFOILILI FSAFSENNES SNMPSSIASF
 201 LSSITYSLYD SIILKGYKRP LTLEDVWEVD EEMKTKTVS KFETHMKREL QKARRALORR QEKSSQONSG ARLPGLNKNO SQSQDALVLE DVEKKKKKSG
 301 TKKDVPKSWL HKALFKTFYM VLLKSFLKL VNDIFTEVSP QLLKLLISFA SORDTYLWIG YLCAILLFTA ALIOSFCLOC YFQLCFKLG VVRTAIMASV
 401 YKALTLSNL ARKEYTVGET VNLMSVDAQK LMDVTNFMHM LWSSVLOIVL SIFFLWREL G PSVLAGVGVM VLVIPINAIL STKSKTIQVK NMKNKDKRLK
 501 IMNEILSGIK ILKYFAWEPS FROQVONLRK KELKNLLAFS QLQCVVIFVF QLTPVLVSUV TFSVYVLVDS NMILDAQKAF TSITLFNHLR FPLSMLPMH
 601 SSMLQASVST ERLEKYLGGD DLDTSAIRHD CNFOKAMQFS EASFTWEHDS EATVRDVNLD IMAGQLVAVI GPVGSCKSSL ISAMLGEMEN VHGHITIKGT
 701 TAYVPOQSWI QNGTIKDNIL FGTEFNKRY QOVLEACALL PDEMLPGGD LAEIGEGIN LSGGOKORIS LARATYCNLD IYLLDOPLSA VOANVGKHF
 801 NKVLGPNGLL KGTKRLVTH SMHFLPOVDE IVVLGNGTIV EKGYSALLA KGEFAKNLK TFLRHTGPEE EATVHOGSEE EDDDYGLISS VEEIPEDAAS
 901 ITMRRENSFR RTLSRSSRN GRHLKSLRNS LKTRNVNSLK EDEELVKGOK LIKKEFIETG KVKFSIYLEY LOAIGLFSIF FIILAFVMNS VAFIGSNLWL

 1001 SAWTSOSKIF NSTDYPASQR DMRVGVYDAL GLAOGIFVFI AHFUSAFGV HASHLHKOL LNNILRAPMR FFDTPGTGRI VNRFAQDIST VDDTLPOSRL
 1101 SWITCFLGII STLVICHAT PVFTIIVIPL GIIYVSVMF YVSTSRQLRR LOSVTRSPIY SHFSETVSGI PVIRAFHQO RFLKHNEERI DTNQCQVSW
 1201 ITSNRWLAIR LELVGNLTVF FSALMMVIYR DILSGDTVGF VLSNALNITO TLNWLVRMTS ELETNIVAVE RITEYTKVEN EAPWVTDKRP PPDWPSKGI
 1301 QFNHYQVRYR PELDLVLRGI TCDIGSMEKI GVVGRTGAGK SSLTNCLFRI LEAGGOTII DGVDIASIGL HDLREKLTII PDDPILFSGS LRMNLDPFNN
 1401 YSDEEIKWAL ELAHLKSFWA SLQLGLSHEV TEAGGNLSIG OROLLCLGRA LLRKSILVL DEATAAVDLE TDNLITOTTIO NEFANCTVIT IAHRLHTIMO
 1501 SOKVMVLONG KIIYGSPEE LLOIPGPFYF MAKEAGIENV NSTKF*

FIG. 10

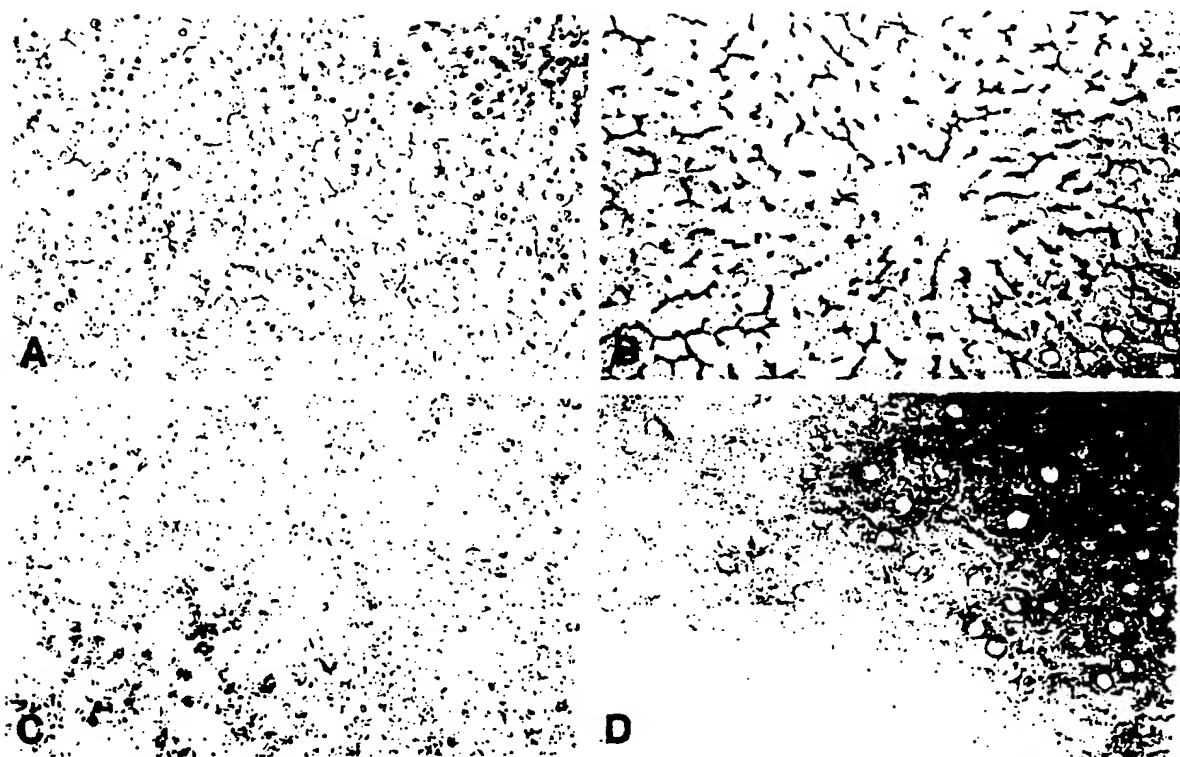


FIG. 11

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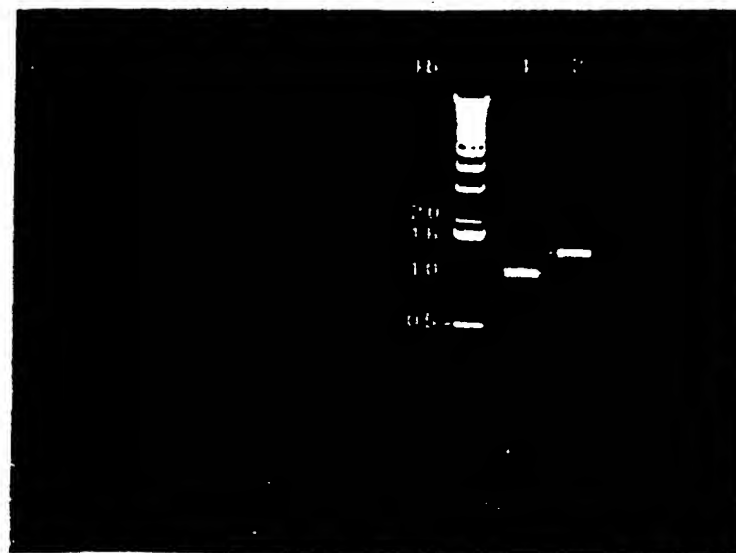
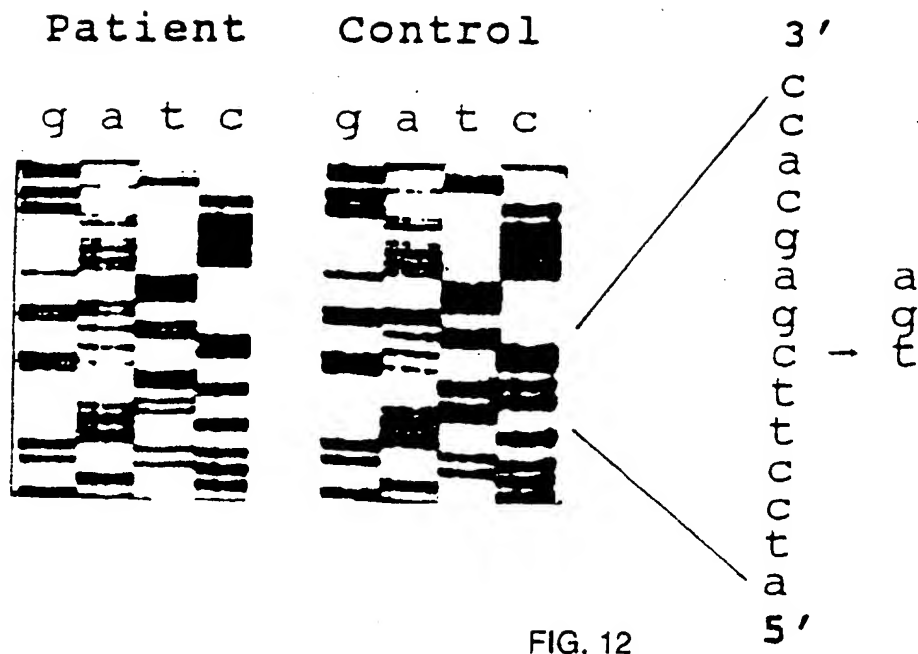


FIG. 13

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| | | | | | | | |
|--------|------|------------|------------|-------------|------------|------------|--------|
| CMOAT/ | MRP1 | FVLRHINVTI | NGGEKVGIVG | RTGAGKSSLT | LGLFRINESA | EGEIIIDGIN | ** * * |
| | MRP2 | LVLRGITCDI | GSMEKIGVVG | RTGAGKSSLT | NCLFRILEAA | GGQIIIDGVD | ** * * |
| | MRP3 | LVLRLSLHV | HGGEKVGIVG | RTGAGKSSMT | LACSRILEAA | KGEIRIDGLN | ** * * |
| | MRP4 |TI | KPKEKIGIVG | RTGSGKSSLG | MAFLRLVELS | GGCIKIDGVR | ** * * |
| | MRP5 |TI | KPKEKIGIVG | RTGSGKSSLG | MAFLRLVELS | GGCIKIDGVR | ** * * |
| | HSUR | PVLKHVNALI | SPGQKIGICG | RTGSGKSSFS | LAFFRMVDTF | EGHIIIDGID | ** * * |
| | MRP1 | IAKIGLHDLR | FKITIIPQDP | VLFSGSLRMN | LDPFQSYSDE | EVWTSLELAH | ** * * |
| | MRP2 | IASIGLHDLR | EKLTIIPQDP | VLFSGSLRMN | LDPFNNYSDE | EIWKALELAH | ** * * |
| | MRP3 | VADIGFHDVR | CQMTIIPRDP | ILFSGTLRMN | LDPFQSYSEE | DIWVALELSH | ** * * |
| | MRP4 | | | | | | ** * * |
| | MRP5 | ISDIGLADLR | SKLSIIPQEP | VLFSGTVRSN | LDPFNQYTED | QIWDALERTH | ** * * |
| | HSUR | IAKLPLHTIR | SRLSIILQDP | VLFSGTIRFN | LDPERKCSDS | TLWEALEIAQ | ** * * |
| | MRP1 | LKOFVSALPD | KLDHECAEGG | ENLSVGQRQL | VCLARALLRK | TKILVLDEAT | ** * * |
| | MRP2 | LKSFVASLQL | GLSHEVTEAG | GNLSIGQRQL | LCLGRALLRK | SKILVLDEAT | ** * * |
| | MRP3 | LHTFVSSQPA | GLDFQCSEGG | ENLSVGQRQL | VCLARALLRK | SRILVLDEAT | ** * * |
| | MRP4 | LKETIEDLPG | KMDTELAESG | SNFSVGQRQL | VCLARAILRK | NOILIIDEAT | ** * * |
| | MRP5 | MKECIAQLPL | KLESEVMENG | DNFSVGQRQL | LCLARALLRH | CKILIIDEAT | ** * * |
| | HSUR | LKLVVKALPG | GLDAIITEGG | ENFSVGQRQL | FCLARAFVRK | TSIFIMDEAT | ** * * |
| | MRP1 | AAVDLETDDL | IQSTIRTQFE | DCTVLTIAHR | LNTIMDYTRV | IVLKGGEIQE | ** * * |
| | MRP2 | AAVDLETDDL | IQSTIRTQFE | DCTVLTIAHR | LNTIMDYTRV | IVLKGGEIQE | ** * * |
| | MRP3 | AAVDLETDDL | IQSTIRTQFE | DCTVLTIAHR | LNTIMDYTRV | IVLKGGEIQE | ** * * |
| | MRP4 | AAVDLETDDL | IQSTIRTQFE | DCTVLTIAHR | LNTIMDYTRV | IVLKGGEIQE | ** * * |
| | MRP5 | AAVDLETDDL | IQSTIRTQFE | DCTVLTIAHR | LNTIMDYTRV | IVLKGGEIQE | ** * * |
| | HSUR | ASIDMATENI | LQKVVMTAPA | DRTVVVTIAHR | VHTILSADLV | IVLKRGAILE | ** * * |
| | MRP1 | YGAPSDLLQ | R.GLPFSMAK | DAGL | | | ** * * |
| | MRP2 | YGSPEELLQI | P.GPFYFMAK | EAGI | | | ** * * |
| | MRP3 | FDSPANLIAA | R.GIFYGMAR | DAGL | | | ** * * |
| | MRP4 | YDEPYVLLQN | KESLFYKMWQ | QLG. | | | ** * * |
| | MRP5 | FDTSPVLLSN | DSSRFYAMFA | AAE. | | | ** * * |
| | HSUR | FDKPEKLLSR | KDSVFASFVR | ADK. | | | ** * * |

FIG. 14

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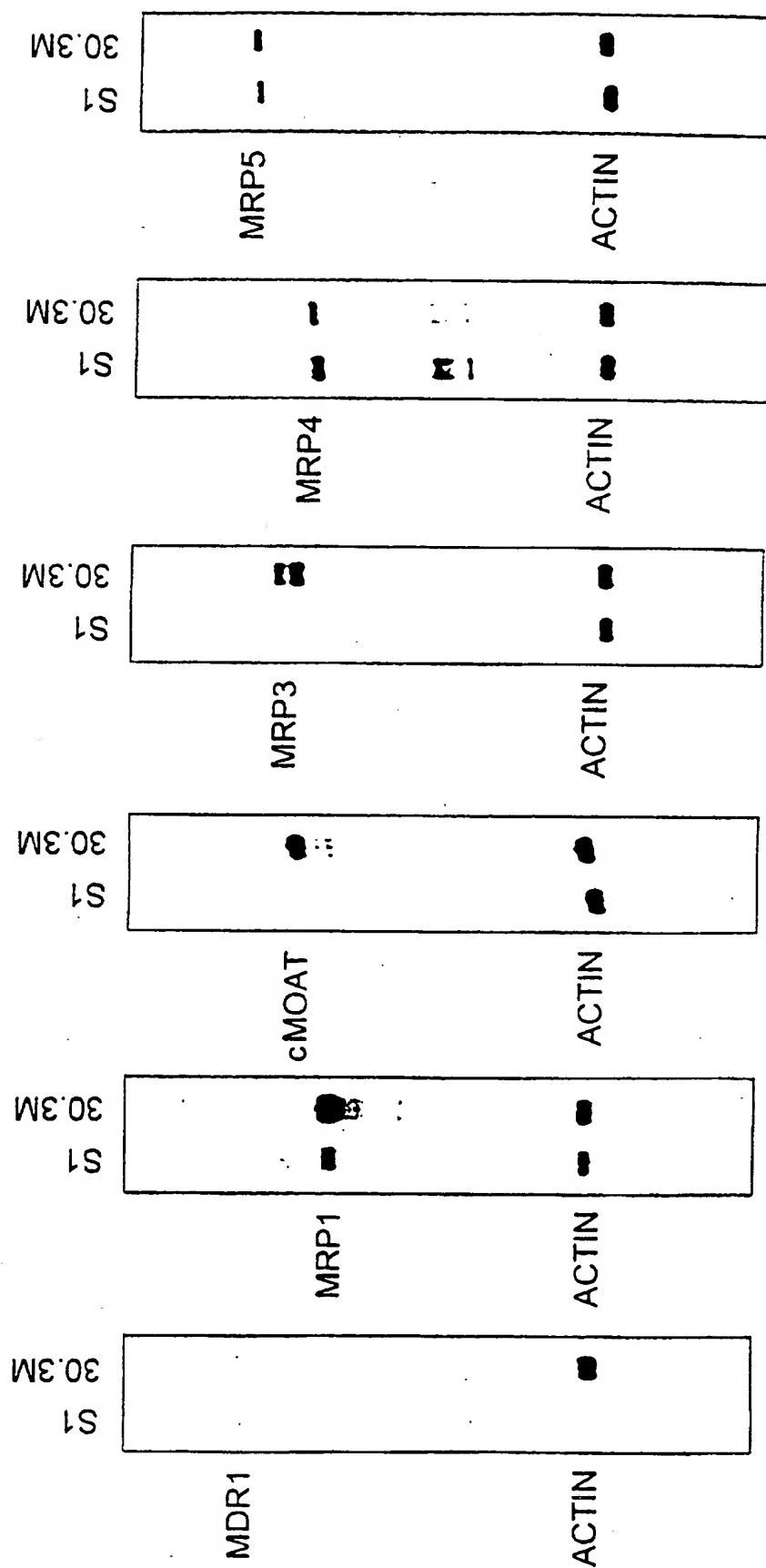


FIG. 15

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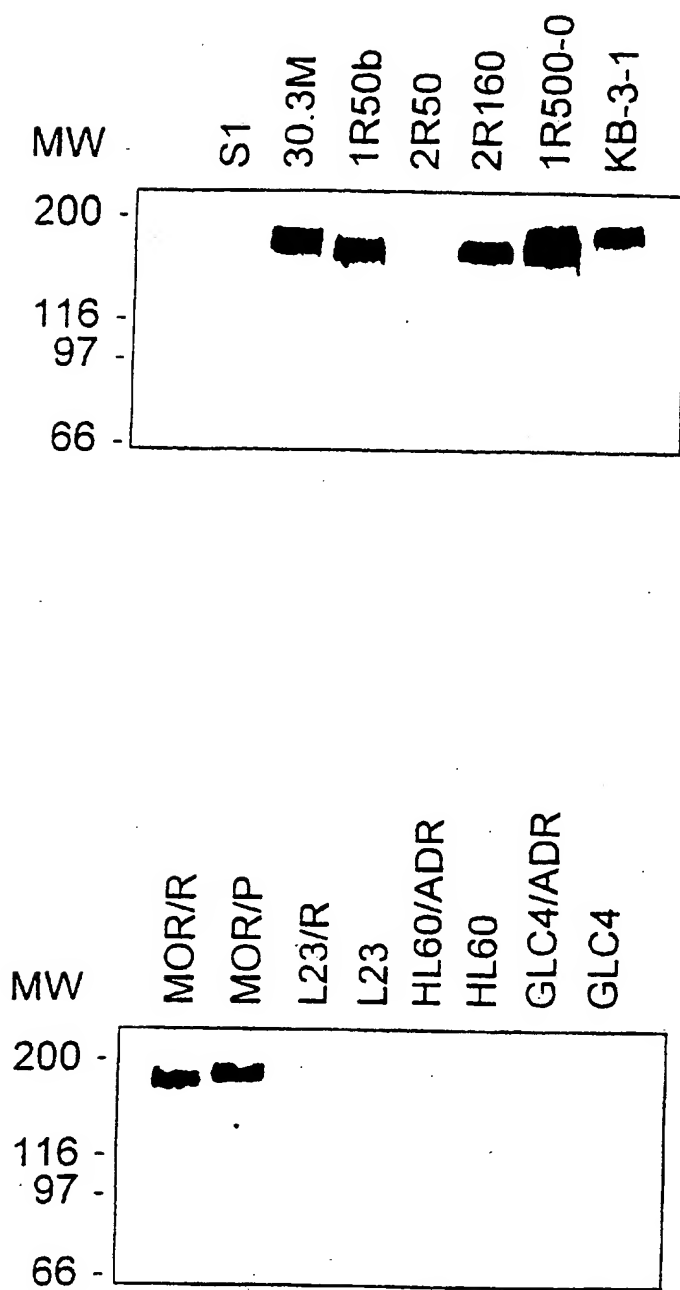


FIG. 16a

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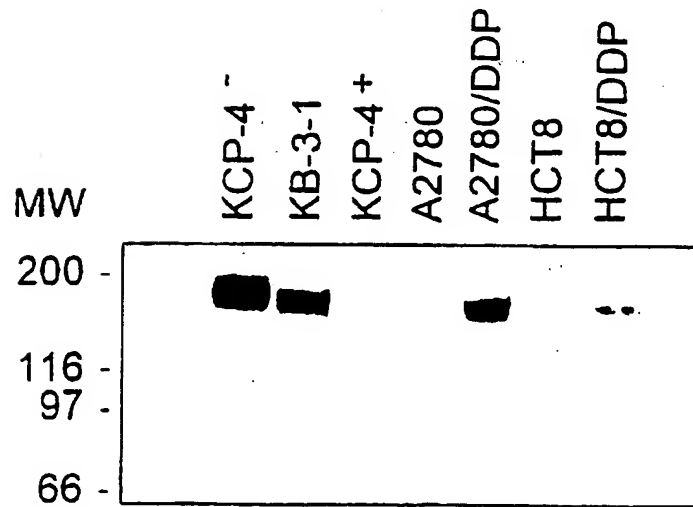
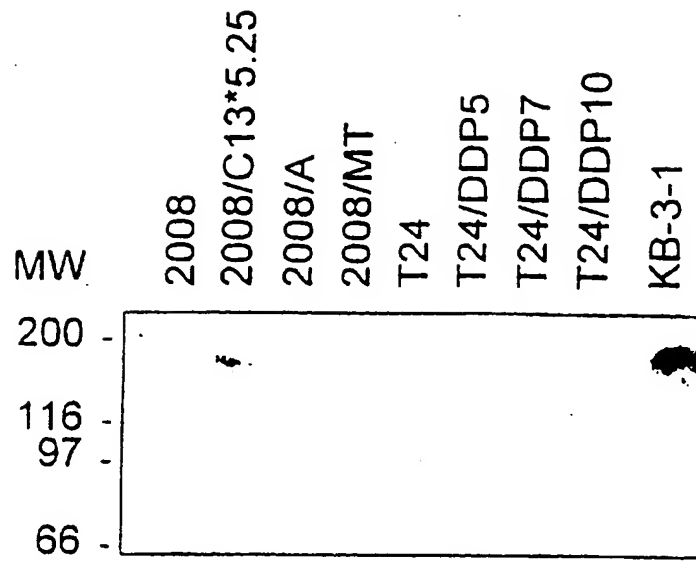


FIG. 16b

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101 GGCTCCAAgt tCTGGGACTC CAACCTGTCT GTGCACACAG AAAACCCGGA
151 CCTCACTCCC TGCTTCCAGA ACTCCCTGCT GGCCTGGGTG CCCTGCATcT
201 ACCTGTGGGT CGCCCTGCCC TGCTACTTGC TcTACcTGCG GCACCATTGT
251 cGTGGCTACA TCATCCTCTC CCACCTGTCC AAGCTCAAGA TGGTCCCTGGG
301 TGTCTGTCTG TGnnnngtgt gctctcctgg cgaccttttt tactcttcca
351 tggcctggtc catggccgcc cctgccccctg ttttctttgt ccccccttg
401 gtggtggggg tcaccatgCT GCTGGCCACC CTGCTGATAC AGTATGAsCG
451 GCTGCAGGGC GTACAGTCTT CGGGGGTCCT CATTATCTTC TGGTTCCTGT
501 GTGTGGTCTG CGCCATCGTC CCATTCCGCT CCAAGATCCT TTAGCCAAG
551 GCAGAGGGTG AGATCTCAGA CCCCTTCCGC TTCACCACCT TCTACATCCA
601 CTTTGCCCTG GTACTCTCTG CCCTCATCTT GGCCTGCTTC AGGGAGAAAC
651 CTCCATTTTT CTCCGCAAAG AATGTCGACC CTAACCCCTA CCCTGAGACC
701 AGCGCTGGCT TTCTCTCCCG CCTGTTTTTC TGGTGGTTCA CAAAGATGGC
751 CATCTATGGC TACCGGCATC CCCTGGAGGA GAAGGACCTC TGGTCCCTAA
801 AGGAAGAGGA CAGATCCAG ATGGTGGTGC AGCAGCTGCT GGAGGCATGG
851 AGGAAGCAGG AAAAGCAGAC GGCACGACAC AAGGCTTCAG CAGCACCTGG
901 GAAAAATGCC TCCGGCGAGG ACGAGGTGCT GCTGGGTGCC CGGCCAGGC
951 CCCGGAAGCC CTCCTTCCTG AAGGCCCTGC TGGCCACCTT CGGCTCCAGC
1001 TTCCTCATCA GTGCCTGCTT CAAGCTTATC CAGGACCTGC TCTCCTTCAT
1051 CAATCCACAG CTGCTCAGCA TCCTGATCAG GTTTATCTCC AACCCCATGG
1101 CCCCCTCCTG GTGGGGCTTC CTGGTGGCTG GGCTTGATGT TCCTGTTGCT
1151 CCATGGATGC AGTCGCTGAT CTTACAACAC TATTACCACT ACATCTTTGT
1201 GACTGGGGTG AAGTTTCGTA CTGGGATCAT GGGTGTcATC TACAGGAAGG
1251 CTCTGGTTAT CACCAACTCA GTCAAACGTG CGTCCACTGT GGGGGAAATT

FIG. 17a

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1301 GTCAACCTCA TGTCA GTGGA TGCCAGCGC TTCATGGACC TTGCCCCCTT
1351 CCTCAATCTG CTGTGGTCAG CACCCCTGCA GATCATCCTG GCGATCTACT
1401 TCCTCTGGCA GAACCTAGGT CCCTCTGTCC TGGCTGGAGT CGCTTTTCATG
1451 GTCTTGCTGA TTCCACTCAA CGGAGCTGTG GCCGTGAAGA TGCGCGCCTT
1501 CCAGGTAAAG CAAATGAAAT TGAAGGACTC GCGCATCAAG CTGATGAGTG
1551 AGATCCTGAA CGGCATCAAG GTGCTGAAGC TGTACGCCTG GGAGCCCAGC
1601 TTCCTGAAGC AGGTGGAGGG CATCAGGCAG GGTGAGCTCC AGCTGCTGCG
1651 CACGGCGGCC TACCTCCACA CCACAACCAC CTTACCTGG ATGTGCAGCC
1701 CCTTCCTGGT GACCCTGATC ACCCTCTGGG TGTACGTGTA CGTGGACCCA
1751 AACAAATGTC TGGACGCCGA GAAGGCCTTT GTGTCTGTGT CCTTGTTTAA
1801 TATCTTAAGA CTTCCCTCA ACATGCTGCC CCAGTTAATC AGCAACCTGA
1851 CTCAGGCCAG TGTGTCTCTG AAACGGATCC AGCAATTCCT GAGCCAAGAG
1901 GAACTTGACC CCCAGAGTGT GGAAAGAAAG ACCATCTCCC CAGGCTATGC
1951 CATCACCATA CACAGTGGCA CTTTACCTG GGCCCAGGAC CTGCCCCCCA
2001 CTCTGCACAG CCTAGACATC CAGGTCCCGA AAGGGGCACT GGTGGCCGTG
2051 GTGGGGCCTG TGGGCTGTGG GAAGTCCTCC CTGGTGTCTG CCCTGCTGGG
2101 AGAGATGGAG AAGCTAGAAG GCAAAGTGCA CATGAAGGGC TCCGTGGCCT
2151 ATGTGCCCCA GCAGGCATGG ATCCAGAACT GCACTCTTCA GGAAAACGTG
2201 CTTTTCGGCA AAGCCCTGAA CCCCAGCGC TAcCAGCAGA CTCTGGAGGC
2251 CTGTGCCTTG CTAGCTGACC TGGAGATGCT GCCTGGTGGG GATCAGACAG
2301 aGATTGGAGA gAAGGGCATT AACCTGTCTG GGGGCCAGCG GCAGCGbGTC
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2451 TCATCGGGCC AGAAGGCGTG CTGGCAGGCA AGACGCGAGT GCTGGTGACC
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2851 GGGTCCATCA GAGAAGGTGC AGGTGACAGA GGCGAAGGCA GATGGGGCAC
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2951 TGGGATTATG CCAAGGCCGT GGGGCTCTGT ACCACGCTGG CCATCTGTCT
3001 CCTGTATGTG GGTCAAAGTG CGGCTGCCAT TGGAGCCAAT GTGTGGCTCA
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3101 CTGAGGCTGG GCGTCTATGC TGCTTTAGGA ATTCTGCAAG GGTCTTGGT
3151 GATGCTGGCA GCCATGGCCA TGGCAGCGGG TGGCATCCAG GCTGCCCCGTG
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3501 GCGGCTCACC TATCTACTCC CACTTTTCGG AGACAGTGAC TGGTGCCAGT
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4201 CTCGGGGACC CTGCGCATGA ACCTGGACCC CTTCGGCAGC TACTCAGAGG
4251 AGGACATTTG GTGGGCTTTG GAGCTGTCCC ACCTGCACAC GTTTGTgAGC
4301 TCCCAGCCGG CAGGCCTGGA CTTCCAGTGC TCAGAGGGCG GGGAGAATCT

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4351 CAGCGTGGGC CAGAGGCAGC TCGTGTGCCT GGCCCGAGCC CTGCTCCGCA
4401 AGAGCCGCAT CCTGGTTTTA GACGAGGCCA CAGCTGCCAT CGACCTGGAG
4451 ACTGACAACC TCATCCAGGC TACCATCCGC ACCCAGTTTG ATACCTGCAC
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4751 CGGCCGGAAT TC

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301 KALLATFGSS FLISACFKLI QDLLSFINPQ LLSILIRFIS NPMAPSWWGF
351 LVAGLDVPA PWMQSLILQH YYHYIFVTGV KFRTGIMGVI YRKALVITNS
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451 PSVLAVAFM VLLIPLNGAV AVKMRAFQVK QMKLKDSRIK LMSEILNGIK
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601 KRIQQFLSQE ELDPQSVERK TISPGYAITI HSGTFTWAQD LPPTLHSLDI
651 QVPKGALVAV VGPVGCCKSS LVSALLGEME KLEGKVHMKG SVAYVPQQAW
701 IQNCTLQENV LFGKALNPKR YQOTLEACAL LADLEMLPGG DQTEIGEKG
751 NLGGQRQRV SLARAVYSDA DIFLLDDPLS AVDSHVAKHI FDHVGPEGV
801 LAGKTRVLVT HGISFLPQTD FIIVLADGQV SEMGPYPALL QRNGSFANFL
851 CNYAPDEDQG HLEDSTALE GAEDKEALLI EDTLSNHTDL TDNDPVTYVV
901 QKQFMRQLSA LSSDGEGQGR PVPRRHLGPS EKVQVTEAKA DGALTQEEKA
951 AIGTVELSVF WDYAKAVGLC TTLAICLLYV GQSAAAIGAN VWLSAWTND
1001 MADSRQNNTS LRLGVYAALG ILQGFLVMLA AMAMAAGGIQ AARVLHQALL
1051 HNKIRSPQSF FDTTPSGRIL NCFSKDIYVV DEVLAPVILM LLNSFFNAIS
1101 TLVVIMASTP LFTVVILPLA VLYTLVQRFY AATSRQLKRL ESVSRSPIYS
1151 HFSETVTGAS VIRAYNRSRD FEIISDTKVD ANQRSCYPYI ISNRWLSIGV
1201 EFVGNVCVLF AALFAVIGRS SLNPGLVGLS VSYSLQVTFA LNMIRMMSD
1251 LESNIVAVER VKEYSKTETE APWVVEGSRP PEGWPPRGEV EFRNYSVRYR

FIG. 17b

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1301 PGLDLVLRDL SLHVHGGEKV GIVGRTGAGK SSMTLCLFRI LEAAKGEIRI
1351 DGLNVADIGL HDLRSQLTII PQDPILFSGT LRMNLDPPGS YSEEDIWWAL
1401 ELSHLHTFVS SQPAGLDFQC SEGGENLSVG QRQLVCLARA LLRKSRIVL
1451 DEATAAIDLE TDNLIQATIR TQFDTCTVLT IAHRLNTIMD YTRVLVLDKG
1501 VVAEFDSPAN LIAARGIFYG MARDAGLA*

mrp4.seq Length: 486 September 26, 1996 09:58 Type: N Check:
879 ..

1 acttaaagaa accattgaag atcttcctgg taaaatggat
actgaattag

51 cagaatcagg atccaatttt agtgttggac aaagacaact
ggtgtgcctt

101 gccagggcaa ttctcaggaa aaatcagata ttgattattg
atgaagcgac

151 ggcaaattgtg gatccaagaa ctgatgagtt aatacaaaaa
aaaatccggg

201 agaaatttgc ccaactgcacc gtgctaacca ttgcacacag
attgaacacc

251 attattgaca gcgacaagat aatggtttta gattcaggaa
gactgaaaga

301 atatgatgag ccgtatgttt tgctgcaaaa taaagagagc
ctattttaca

351 agatggtgca acaacttggc aaggcagaag nnnnnnnct
cactgaaaca

401 gcaaaacngg tatacttcaa agaaaattnt ncacatattg
gtgacnctgn

451 ccacatgggtt acaaacngtt ncaatggncn nnnntc

FIG. 18a

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mrp5a.con Length: 1761 September 10, 1996 11:56 Type: N
Check: 5118 ..

1 CATTGCAATC AGTGGAACCT TCGCTTATGT GGCCCAGCAG
GCTGGATCCT

51 CAATGCTACT CTGAGAGACA ACATCCTGTT TGGGAAGGAA
TATGATGAAG

101 AAAGATACAA CTCTGTGCTG AACAGCTGCT GCCTGAGGCC
TGACCTGGCC

151 ATTCTTCCCA GCAGCGACCT GACGGAGATT GGAGAGCGAG
GAGCCAACCT

201 GAGCGGTGGG CAGCGCCAGA GGATCAGCCT TGCCCGGGCC
TTGTATAGTG

251 ACAGGAGCAT CTACATCCTG GACGACCCCC TCAGTGCCTT
AGATGCCCCAT

301 GTGGGCAACC ACATCTTCAA TAGTGCTATC CGGAAACATC
TCAAGTCCAA

351 GACAGTTCTG TTTnnnGTTA CCCACCAGTT ACAGTACCTG
GTTGACTGTG

401 ATGAAGTGAT CTTCATGAAA GAGGGCTGTA TTACGGAAAG
AGGCACCCAT

451 GAGGAACTGA TGAATTTAAA TGGTGACTAT GCTACCATTT
TTAATAACCT

501 GTTGCTGGGA GAGACACCGC CAGTTGAGAT CAATTCAAAA
AAGGAAACCA

551 GTGGTTCACA GAAGAAGTCA CAAGACAAGG GTCCTAAAAC
AGGATCAGTA

601 AAGAAGGAAA AAGCAGTAAA GCCAGAGGAA GGGCAGCTTG
TGCAGCTGGA

651 AGAGAAAGGG CAGGGTTCAG TGCCCTGGTC AGTATATGGT
GTCTACATCC

701 AGGCTGCTGG GGGCCCCTTG GCATTTCTTG GTTATTATGG
CCCTTTTCAT

751 GGCCTGAATG TAGGCAGCAC CGCCTTCAGC ACCTGGTGGT
TGAGTTACTG

801 GATCAAGCAA GGAAGCGGGA ACACCACTGT GACTCGAGGG
AACGAGACCT

FIG. 19a

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851 CGGTGAGTGA CAGCATGAAG GACAATCCTC ATATGCAGTA
CTATGCCAGC

901 ATCTACGCCC TCTCCATGGC AGTCATGCTG ATCCTGAAAG
CCATTCGAGG

951 AGTTGTCTTT GTCAAGGGCA CGCTGCGAGC TTCCTCCCGG
CTGCATGACG

1001 AGCTTTTCCG AAGGATCCTT CGAAGCCCTA TGAAGTTTTT
TGACACGACC

1051 CCCACAGGGA GGATTCTCAA CAGGTTTTCC AAAGACATGG
ATGAAGTTGA

1101 CGTGCGGCTG CCGTTCCAGG CCGAGATGTT CATCCAGAAC
GTTATCCTGG

1151 TGTTCCTCTG TGTGGGAATG ATCGCAGGAG TCTTCCCGTG
GTTCCTTG TG

1201 GCAGTGGGGC CCCTTGTCAT CCTCTTTTCA GTCCTGCACA
TTGTCTCCAG

1251 GGTCTGATT CGGGAGCTGA AGCGTCTGGA CAATATCACG
CAGTCACCTT

1301 TCCTCTCCCA CATCACGTCC AGCATAACAGG GCCTTGCCAC
CATCCACGCC

1351 TACAATAAAG GGCAGGAGTT TCTGCACAGA TACCAGGAGC
TGCTGGATGA

1401 CAACCAAGCT CCTTTTTTTT TGTTTACGTG TGCGATGCGG
TGGCTGGCTG

1451 TGCGGCTGGA CCTCATCAGC ATCGCCCTCA TCACCACCAC
GGGGCTGATG

1501 ATCGTTCTTA TGCACGGGCA GATTCCCCCA GCCTATGCGG
GTCTCGCCAT

1551 CTCTTATGCT GTCCAGTTAA CGGGGCTGTT CCAGTTTACG
GTCAGACTGG

1601 CATCTGAGAC AGAAGCTCGA TTCACCTCGG TGGAGAGGAT
CAATCACTAC

1651 ATTAAGACTC TGTCCCTTGA AGCACCTGCC AGAATTAAGA
ACAAGGCTCC

1701 CTCCCCTGAC TGGCCCCAGG AGGGAGAGGA agAaGGTGAA
CstTTTtGAGA

1751 AAGGGGCCTT T

FIG. 19a-2

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mrp5b.con Length: 2167 November 19, 1996 14:33 Type: N
Check: 6982 ..

1 TTATGACCMc tGrAnTrrCg CCmAGGGGGs AgAkkrACCn
TTcaTkAgAA

51 GGaaCArAaG aTGgGTACsG AgAAaCtTCc tttTTaTCc
tAaAagaaAg

101 TATyCtttCA CGATCAAACC TAAAGAGAAG ATTGGCATTG
TGGGGCGGAC

151 AGGATCAGGG AAGTCCTCGC TGGGGATGGC CCTCTCCGT
CTGGTGGAGT

201 TATCTGGAGG CTGCATCAAG ATTGATGGAG TGAGAATCAG
TGATATTGGC

251 CTTGCCGACC TCCGAAGCAA ACTCTCTATC ATTCCTCAAG
AGCCGGTGCT

301 GTTCAGTGGC ACTGTCAGAT CAAATTGGA CCCCTTCAAC
CAGTACACTG

351 AAGACCAGAT TTGGGATGCC CTGGAGAGGA CACACATGAA
AGAATGTATT

401 GCTCAGCTAC CTCTGAAACT TGAATCTGAA GTGATGGAGA
ATGGGGATAA

451 CTTCTCAGTG GGGGAACGGC AGCTCTTG TG CATAGCTAGA
GCCCTGCTCC

501 GCCACTGTAA GATTCTGATT TTAGATGAAG CCACAGCTGC
CATGGACACA

551 GAGACAGACT TATTGATTCA AGAGACCATC CGAGAAGCAT
TTGCAGACTG

601 TACCATGCTG ACCATTGCCC ATCGCCTGCA CACGGTTCTA
GGCTCCGATA

651 GGATTATGGT GCTGGCCCAG GGACAGGTGG TGGAGTTTGA
CACCCCATCG

701 GTCCTTCTGT CCAACGACAG TTCCCGATTC TATGCCATGT
TTGCTGCTGC

751 AGAgAACAAG GTCGCTGTCA AGGGCTGACT CCTCCCTGTT
GACGAAGTCT

801 CTTTTCTTTA GAGCATTGCC ATTCCCTGCC TGGGGCGGGC
CCCTCATCGC

FIG. 19b

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851 GTCCTCCTAC CGAAACCTTG CCTTTCTCGA TTTTATCTTT
CGCACAGCAG

901 TTCCGGATTG GCTTGTGTGT TTCACTTTTA GGGAGAGTCA
TATTTTGATT

951 ATTGTATTTA TTCCATATTC ATGTAAACAA AATTTAGTTT
TTGTTCTTAA

1001 TTGCACTCTA AAAGGTTTCAG GGAACCGTTA TTATAATTGT
ATCAGAGGCC

1051 TATAATGAAG CTTTATACGT GTAGCTATAT CTATATATAA
TTCTGTACAT

1101 AGCCTATATT TACAGTGAAA ATGTAAGCTG TTTATTTTAT
ATTAAAATAA

1151 GCACTGTGCT AATAACAGTG CATATTCCTT TCTATCATTT
TTGTACnGTT

1201 TGCTGTACnA nAAATCTGGT nTTGCTmTTm nACTGTTAGG
AAGAATTAnC

1251 ATTTCATTCT TCTCTAgCTG GTGGTTtCAC gGTGgCCAGG
TTTTCTGGGT

1301 GTCCAAAGGA AGACGTGTtG GCAATAGTtn GGGCCCTCCG
ACAAGCCCCC

1351 TCTGCCGCCT CCCACAGCC GCTCCAnGGG GTGGCTGGAG
AaCGGGTGGG

1401 CGGCTGGAGA CCATGCCAGA GCGCCGTGAG TTCTCAGGGC
TCCTGCCTTC

1451 TGTCCCTGGTG TCACTTACTG TTTCTGTtCA GGgAGAGCAG
CGGGGCGAAG

1501 CCCAGGCCCC TTTTCACTCC CTCCATCAAG AATGGGGATC
ACAGAGACAT

1551 TCCTCCGAGC CGGGGAGTTT CTTTCCTGCC TTCTTCTTTT
TGCTGTTGTT

1601 TCTAAACAAG AATCAGTCTA TCCACAGAGA GTCCCACTGC
CTCAGGTTCC

1651 TATGGCTGGC CACTGCACAG AGCTCTCCAG CTCCAAGACC
TGTTGGTTCC

1701 AAGCCCTGGA GCCAACTGCT GCTTTTTGAG GTGGCACTTT
TTCATTGCC

FIG. 19b-2

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1751 TATTCCCACA CCTCCACAGT TCAGTGGCAG GGCTCAGGAT
TTCGTGGGTC

1801 TGTTTTTCCTT TCTCACC GCA GTCGTCGCAC AGTCTCTCTC
TCTCTCTCCC

1851 CTCAAAGTCT GCAACTTTAA GCAGCTCTTG CTAATCAGTG
TCTCACACTG

1901 GCGTAGAAGT TTTTGTACTG TAAAGAGACC TACCTCAGGT
TGCTGGTTGC

1951 TGTGTGGTTT GGTGTGTTCC CGCAAACCCC CTTTGTGCTG
TGGGGCTGGT

2001 AGCTCAGGTG GGCGTGGTCA CTGCTGTCAT CAGTTGAATG
GTCAGCGTTG

2051 CATGTCGTGA CCAACTAGAC ATTCTGTCGC CTTAGCATGT
TTGCTGAACA

2101 CCTTGTGGAA GCAAAAATCT GAAAATGTGA ATAAAATTAT
TTTGGATTTT

2151 GTAAAAAAA AAAAAA

FIG. 19b-3



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|--|
| (51) International Patent Classification ⁶ : C12N 15/12, C07K 14/705, C12Q 1/68, A61K 48/00 | A3 | (11) International Publication Number: WO 97/31111 (43) International Publication Date: 28 August 1997 (28.08.97) |
| (21) International Application Number: PCT/NL97/00079 (22) International Filing Date: 21 February 1997 (21.02.97) (30) Priority Data: 96200460.2 22 February 1996 (22.02.96) EP (34) Countries for which the regional or international application was filed: NL et al. (71) Applicants (for all designated States except US): INTROGENE B.V. [NL/NL]; Lange Kleiweg 151, NL-2288 GJ Rijswijk (NL). ACADEMISCH MEDISCH CENTRUM AMSTERDAM [NL/NL]; Meibergdreef 9, NL-1105 AZ Amsterdam (NL). HET NEDERLANDS KANKER INSTITUUT [NL/NL]; Plesmanlaan 121, NL-1066 CX Amsterdam (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): OUDE ELFERINK, Ronald, Petrus, Johannes [NL/NL]; Dr. Koomansstraat 27, NL-1391 XA Abcoude (NL). PAULUSMA, Coenraad, Cornelis [NL/NL]; Daendelsstraat 25 bis, NL-3531 GB Utrecht (NL). BOSMA, Piter, Jabik [NL/NL]; Gildstraat 124, NL-3572 ES Utrecht (NL). BORST, Piet [NL/NL]; Meentweg 87, NL-1406 KE Bussem (NL). EVERS, Raymond [NL/NL]; Berlaarstraat 57, NL-1066 PJ Amsterdam | | (NL). KOOL, Marcel [NL/NL]; Van Walbeekstraat 52-I, NL-1058 CV Amsterdam (NL). (74) Agent: SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 27 November 1997 (27.11.97) |
| (54) Title: A FAMILY OF ORGANIC ANION TRANSPORTERS, NUCLEIC ACIDS ENCODING THEM, CELLS COMPRISING THEM AND METHODS FOR USING THEM (57) Abstract <p>The present invention provides a novel family of organic anion transporters of which until now only one member was known. The family includes multispecific organic anion transporters related to the canalicular multispecific organic anion transporter. The cDNA encoding the latter is also provided. The rat and human cDNA are exemplified. Uses of nucleic acids based on this gene family and of cells comprising such nucleic acids as well as vectors comprising sequences thereof are also disclosed especially in the area of gene therapy.</p> | | |

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 97/00079

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/705 C12Q1/68 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| X | MAYER R ET AL: "Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes." J CELL BIOL, OCT 1995, 131 (1) P137-50, UNITED STATES, XP000608636 see the whole document --- | 1-6,20, 21 |
| X | COLE SP ET AL: "Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line [see comments]" SCIENCE, DEC 4 1992, 258 (5088) P1650-4, UNITED STATES, XP002017513 see the whole document --- -/-- | 1-6,20, 21 |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

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- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
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- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- *&* document member of the same patent family

Date of the actual completion of the international search

10 July 1997

Date of mailing of the international search report

21.10.97

Name and mailing address of the ISA

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Authorized officer

Gurdjian, D

INTERNATIONAL SEARCH REPORT

Intel. Patent Application No.

PCT/NL 97/00079

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| X | ZAMAN GJ ET AL: "The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump." PROC NATL ACAD SCI U S A, SEP 13 1994, 91 (19) P8822-6, UNITED STATES, XP002017514 | 1-7, 11-17, 20,21 |
| Y | see the whole document --- | 8-10 |
| X | WO 94 10303 A (UNIV KINGSTON) 11 May 1994 see claims 1-80; figures SEQ.1,2 --- | 1-22 |
| Y | WO 92 12987 A (US) 6 August 1992 see page 32, line 26 - page 33, line 22 --- | 8-10 |
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| P,X | PAULUSMA CC ET AL: "Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene." SCIENCE, FEB 23 1996, 271 (5252) P1126-8, UNITED STATES, XP002017518 see the whole document ----- | 1-6,20, 21 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NL 97/ 00079

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 23 and claims 16-18, partially as far as they concern an in vivo method, are directed to a method of treatment of the human/animal body, a search has been carried out based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-24 all partially (Subject 1.)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/NL 97 00079

FURTHER INFORMATION CONTINUED FROM PCT/ISA210

Subject 1: Claims 1-24 (partially): the canicular multispecific organic anion transporter cMOAT protein and gene.

Subject 2: Claims 1-24 (partially): the multidrug resistant-associated protein MRP3 protein and gene.

Subject 3: Claims 1-24 (partially): the multidrug resistant-associated protein MRP4 protein and gene.

Subject 4: Claims 1-24 (partially): the multidrug resistant-associated protein MRP5 protein and gene.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 97/00079

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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